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(2)	Method for the microbiological preparation of human serum albumin and other heterologous proteins from a yeast.	(7) Microbiological method for the preparation of human serum albumin and other heterologous (non-yeast) proteins by growing a yeast, especially of the genus <u>Kluyveromyces</u> , modified by the use of recombinant DNA techniques.

EP 0 361 991 A2

METHOD FOR THE MICROBIOLOGICAL PREPARATION OF HUMAN SERUM ALBUMIN: AND OTHER HETEROLOGOUS PROTEINS FROM A YEAST

The present invention relates to a microbiological method for the preparation of human serum albumin (HSA) by culturing a yeast, especially of the genus Kluyveromyces, modified by the use of recombinant DNA techniques.

HSA is a protein of 585 amino acids with a molecular weight of 68 kilodaltons (kD), taking the form of a globular, unglycosylated monomer. Its globular structure is maintained by 17 disulfide bonds which create a sequential series of 9 double loops (Brown, J.R., in "Albumin Structure, Function and Uses", Rosemarie, V.M. et al. (eds.), Pergamon Press, Oxford, (1977) 27 - 51). The genes encoding HSA are known to be highly polymorphic, and more than 30 apparently different genetic variants have been typed by electrophoretic analysis under various conditions (Weikamp, L.R. et al., Ann. Hum. Genet. 37 (1973) 219 - 226).

The HSA gene is split into 15 exons with 14 intron sequences, and comprises more than 16.9 kilobases (kb) from the putative "Cap" site to the first poly(A) addition site.

Human albumin is synthesized in the liver hepatocytes, from which it is secreted into the blood-stream. It is the most abundant protein in the blood, with a concentration of about 40 g/liter of serum; there are hence about 160 g of circulating albumin in the human body at any time. The most important role of HSA is to maintain normal osmolarity in the bloodstream. It also has an exceptional binding capacity for various substances and drugs, in addition, a role in the endogenous transport of hydrophobic molecules (e.g. steroids and biliary salts) as well as of various therapeutic substances which can thus be transported to their respective action sites. Furthermore, HSA has recently been implicated in the catabolism of pro-tropolectins.

The synthesis of HSA in the hepatocytes first yields a precursor, pre-pro-HSA, which contains a signal sequence of 18 amino acids directing the nascent polypeptide to the secretory pathway. This signal sequence is cleaved off, probably by cotranslational processing, before the protein is released from the endoplasmic reticulum. This first proteolytic cleavage yields the precursor pro-HSA, which still contains a N-terminal hexapeptide (Arg-Gly-Val-Phe-Arg-Arg) not normally present in the mature form of circulating HSA. A convertase, probably located in the Golgi vesicles removes the pro-peptide in a second proteolytic step by cleaving the hexapeptide bound to the N-terminal acidic acid of mature HSA (Judah, J.D. and Quinn, P.L., Nature 271 (1977) 384 - 385; Bathurst, I.C. et al., Science 235 (1987) 348 - 350). However, non-processed human albumin may represent half of that present in the bloodstream in rare heterozygous individuals who carry an inherited mutation in the pro-peptide sequence, and all of the albumin in some extremely rare homozygotes (Takahashi, N. et al., Proc. Natl. Acad. Sci. USA 74 (1987) 7403 - 7407). One of these variants is designated human Christchurch and begins with the sequence Arg-Gly-Val-Phe-Arg-Gln-Asp⁻, in which the substitution Arg⁻...>Gln prevents the cleavage of the pro-peptide thus mutated (Brennan, S.O. and Carrel, R.W., Nature 274 (1978) 908 - 909). Other variants known as pro-albumin Lille (Abdo, Y. et al., FEBS Letters 131 (1981) 285 - 288) and Takao (Nagata et al., Rinsho Byori 30 (1982) 791 - 796) have the following N-terminal sequences, respectively: Arg-Gly-Val-Phe-Arg-His-Asp and Arg-Gly-Val-Phe-Arg-Pro-Asp. In each of these cases, the observed mutation affects the pair of basic amino acids Arg-Arg which normally precedes the amino-acid sequence of mature human albumin, and results from a single-base change in the arginine codon (Takahashi, N. et al., Proc. Natl. Acad. Sci. USA 84 (1987) 7403 - 7407).

Cleavage after a pair of basic amino acids is an essential feature of the maturation of other proteins, including peptide hormones, neuropeptides and plasma proteins other than HSA (Dougherty, O. et al., Annals Rev. Biochem. 53 (1984) 665 pp.). Recently, it has been disclosed that the convertase which cleaves pro-albumin to albumin in the liver is probably very closely related to the protease *yscF* of yeast (Bathurst, I.C. et al., Science 235 (1987) 348 - 350). This calcium-dependent metalloprotease is encoded by the *KEK2* gene of Saccharomyces cerevisiae, and is probably bound to the membrane of the Golgi apparatus. It is known to be involved in the maturation of the pheromone referred to as alpha-factor and in that of the killer toxin, since *kek2* mutants of S. cerevisiae are incapable of cleaving the respective pro-proteins at the pair of basic amino acids Lys-Arg (Junius, D.J. et al., Cell 32 (1983) 839 pp.). Furthermore, it can be demonstrated that the yeast enzyme encoded by the *KEK2* gene correctly recognizes and cleaves the normal pro-sequence of Saccharomyces cerevisiae, in vitro, but does not cleave the mutated pro-sequence (Arg-Gln) of pro-albumin (*Arg-Arg*) or albumin *in vivo*, and that the killer toxin produced has recently been described as being encoded by the *KEK1* gene of K. lactis (Węsławski-Louvel, M. et al., Yeast 4 (1988) 71 - 81). In the light of this finding, the capacity of these yeasts to mature recombinant human albumin correctly and to secrete the latter into the culture medium was tested.

Albumin accounts for 40% of the world market in plasma proteins. Commercial interest in it stems from

the fact that this product is widely used, for example, in so-called blood-volume expanders to compensate for blood losses during surgery, accidents, hemorrhages, and at doses per day and per individual in the multi-gram range.

Up to now, HSA is generally produced by conventional techniques of fractionation of plasma derived from blood donors (Cohn, E.J. et al., J. Am. Chem. Soc. 68 (1946) 459 pp.), the worldwide annual consumption of plasma being in the region of 9.5 x 10⁶ litres. It can also be extracted from human placenta according to the technique described by J. Lautaud et al. (13th Intern. Congress of IABS, Budapest: A.

"Purification of proteins. Development of biological standard", Karger (ed.), Bale, 27 (1973) 107 pp.). The development of genetic engineering and of new extraction and purification techniques has opened up the possibility of obtaining improved products of higher purity and better stability without viral contamination (e.g. hepatitis B and AIDS) and at lower cost. However, no procedure based on genetic engineering is known as yet which is efficient enough to be of economic interest for the industrial-scale production of HSA.

This is mainly due to the lack of an efficacious host/vector system which would permit the high-level production of a correctly matured secreted albumin possessing proper tertiary structure and having the physicochemical properties of native human albumin.

Even though the use of mammalian cell cultures might appear the ideal choice for the expression of human proteins, the cost of such a procedure would be well beyond the current selling price of albumin used for pharmaceutical purposes (Klauser, A., Biotechnology 3 (1985) 119 - 125). Since this product is generally prescribed in multi-milligram quantities and at a low price, biotechnological production of HSA seems to be feasible only with microbial fermentation systems.

Until recently, the vast majority of genetic engineering experiments have employed Escherichia coli as the host organism for the microbial production of heterologous proteins of economic importance. Even though this type of procedure has appeared to be satisfactory for a number of heterologous proteins, all attempts to produce HSA under economically acceptable conditions using this organism have been only partially successful.

One of the major drawbacks associated with the use of E. coli lies in the fact that this bacterium is, in most cases, incapable of excelling the heterologous protein into the culture medium, and that these proteins accumulate in one of the cellular compartments. The desired protein must hence be separated from cellular matter, giving rise to complex and expensive procedures. Furthermore, E. coli produces endotoxins and pyrogens which can contaminate the proteins produced. For this reason, great care must be taken in the purification to remove residual endotoxins in the end product, especially when the latter is used as a medicinal product.

Secreted proteins often lose the property of folding correctly if they are synthesized and accumulated in the cytoplasm. In general, secretion is required in order to make possible the formation of disulfide bonds such as those present in HSA. The fact that HSA produced in E. coli is not secreted thus causes it to precipitate intracellularly in an insoluble form (Latta, M. et al., Biotechnology 5 (1987) 1309 - 1314). Consequently, after extraction from the bacterial cells, the protein has to be denatured and then renatured *in vitro*.

Furthermore, E. coli lacks the cellular machinery making it possible to perform the post-transcriptional and post-translational modifications specific to eukaryotic organisms, including yeasts and humans. With regard to HSA, it appears to be difficult to induce E. coli to express at a satisfactory level the natural HSA precursor, i.e. pre-pro-HSA. Moreover, artificial albumins in which the HSA structural gene has been fused to secretion signals derived from the bacterial *pac* or *ompA* genes are generally processed incompletely (ibid.). Furthermore, the expression of HSA without the pre-sequence, i.e. in the form Met-HSA, shows that the N-terminal methionine residue is not excised by the E. coli methionine aminopeptidase (MAP) (ibid.; European patent application EP 198 745, publ. 22.10.1986). In consequence, E. coli-derived HSA cannot be obtained in mature form *in vivo* and must be modified *in vitro*, by trypic cleavage, in order to excise a phage-derived leader sequence, subsequent to denaturation and renaturation *in vitro* (European patent application EP 238 210, publ. 08.09.1987).

As regard other bacterial hosts, studies have recently been published on the secretion of HSA by Bacillus subtilis (Saunders, C.W. et al., J. Bacteriol. 169 (1987) 2917 - 2925). Although this study indicates that a prokaryotic organism such as B. subtilis possesses the potential for secreting high molecular weight human proteins such as HSA, it also shows that excision in the growth medium can be obtained only when using B. subtilis protoplasts, i.e. bacterial cells whose normal cell wall has been digested by enzymatic treatment. Furthermore, the percentage of modified HSA is inversely proportional to the level of protein produced: at high expression levels, most of the recombinant HSA remains in unmodified form (ibid.).

Moreover, no data concerning the physicochemical properties of the recombinant HSA from B. subtilis have

been reported. Furthermore, the level of HSA secretion obtained by this microorganism is very low in the light of the amounts of HSA detected in the culture supernatants using immunological methods (European patent application EP 0 229 712 A2, publ. 22.07.1987).

An attractive alternative to the use of bacterial hosts for the preparation of heterologous proteins is the use of microbial eukaryotic systems such as yeasts or fungi. In effect, these organisms possess all the features of structure and cellular organization found in more complex eukaryotic organisms such as mammalian cells. In particular, yeasts are capable of carrying out the post-transcriptional and post-translational modifications which are important for the activity of many proteins. In addition, yeasts are very common in industrial-scale production, can be grown to high cell densities, are not pathogenic, do not produce endotoxins and have been used in the food industry since ancient times. Finally, in contrast to mammalian cells, genetic manipulations with yeasts are easy to perform, and classical and molecular genetics have provided a large body of data.

The term yeast is frequently used to indicate Saccharomyces cerevisiae, or baker's yeast, which is one of the most common and best known species. It is understood hereinafter that the term yeast applies to other genera, and is not restricted to the species S. cerevisiae. The expression of pre-pro-HSA under the control of the chitinase promoter, has been demonstrated in S. cerevisiae at maximal levels of about 1 % of total proteins (Etcheverry, T. et al., BioTechnology 4 (1986) 726 - 730). However, the material recognized by anti-HSA antibodies which is described in this study remains cell-associated, and therefore is not truly exported into the culture supernatant. In addition, no detailed characterization of the recombinant protein has been reported.

Production of HSA in brewer's yeast using a post-fermentation process during beer brewing has also been reported (European patent application EP 0 201 239, publ. 12.11.1986). Once again, no quantitative or qualitative data concerning the product obtained has been described. Furthermore, this process leads to the expression of Met-HSA, i.e. an allele of HSA starting with the amino acid methionine just upstream from the sequence of mature albumin. The absence of a signal sequence precludes the secretion and maturation of the recombinant HSA, and causes accumulation of an intracellular albumin whose tertiary structure has not been characterized. In addition, the absence of N-terminal methionine in the final product has not been demonstrated.

The present invention describes the production of genetically engineered, modified yeast strains which can be grown in culture for mass production and which are capable of efficiently producing and excreting HSA in its native conformation into the growth medium.

A preferred expression system involves yeast of the genus Kluyveromyces as a host and uses vectors derived from the natural K. marxianus var. drosophilarium plasmid pkD1.

Moreover, research performed with the object of producing albumin has revealed the value and importance of certain pkD1 sequences in the efficacy and especially the stability of the expression plasmids incorporating them.

The present invention hence also relates to the use of these essential elements of pkD1 in the expression of proteins other than albumin, in particular tissue plasminogen activator (TPA), metalloprotease inhibitor (TIMP) and interleukins, e.g. interleukin-1 α (β), these proteins to be considered only as examples for the purpose of demonstrating that the use of pkD1 plasmid derivatives is "generic", i.e. hardly dependent, if at all, on the nature of the proteins whose expression and/or secretion is desired.

Yeasts of the genus Kluyveromyces and in particular K. marxianus (including the varieties lactis and marxianus which will hereinafter be referred to as K. lactis and K. fragilis, respectively) are important organisms and of considerable commercial interest in the biotechnology industry. K. lactis and K. fragilis are used, for example, for the commercial production of the enzyme lactase (β-galactosidase). These yeasts are capable of growth on whey, a major by-product of the dairy industry. Several Kluyveromyces strains are used for the large-scale production of "single cell protein" (SCP) which play an important role in livestock feeds. Finally, organisms of the genus Kluyveromyces are mentioned on the so-called GRAS list (Generally Recognized As Safe), which represents an important factor for the production of pharmaceutical grade products.

Kluyveromyces have only recently been developed. Three types of cloning vectors have been described for this organism:

- integrating vectors containing sequences homologous to regions of the Kluyveromyces genome and which, after being introduced into the cells, are integrated in the Kluyveromyces chromosomes by vivo recombination. Integration, a very rare event requiring the presence of an efficient selection marker, is obtained when these vectors do not contain sequences permitting autonomous replication in the cell. The advantage of this system is the stability of the transformed strains, i.e. the fact that they can be grown in a normal nutrient support without the need for a selection pressure for maintenance of the integrated plasmid of S. cerevisiae, the proteins encoded by two of these genes (B and C) are probably involved in

sequences. The disadvantage is, however, that the integrated genes are only present in one or, at best, a small number of copies per cell. This low gene dosage often results in a low level of production of a heterologous protein.

- replicating vectors containing autonomously replicating sequences (ARS), derived from the chromosomal DNA of Kluyveromyces sp. (Das, S. and Hollenberg, C.P., Current Genetics 6 (1982) 123 - 128; International patent application WO 83/04050, publ. 24.11.1983; International patent application WO 83/04051, publ. 24.11.1983). Such vectors are of only moderate interest, since their segregation in mitotic cell division is very unhomogeneous, which results in their loss from the cells at a high frequency even when the latter are grown under selection pressure.
- replicating vectors derived from naturally occurring yeast plasmids, either from the linear killer plasmid pGK1-1 (k1) isolated from K. lactis (de Louvencourt, L. et al., J. Bacteriol. 154 (1982) 737 - 742; European patent application EP 0 055 986, publ. 07.12.1983), or from the circular plasmid pkD1 isolated from K. marxianus var. drosophilarium (European patent application EP 0 241 435 A2, publ. 14.10.1987). Vectors containing replication sequences derived from the linear killer plasmid of Kluyveromyces do not have any practical utility for mass production of heterologous proteins since a special nutrient medium is required for their maintenance and since they are lost in 40-99% of the cells in a given population after only 15 generations, even under selection pressure (European patent application EP 0 095 986, publ. 07.12.1983). The most efficient vector system for the transformation of the genus Kluyveromyces described to date is derived from the endogenous plasmid pkD1: constructions containing the entire sequence of pkD1 can be transferred with high frequency, are present in the cell in 70-100 copies and, most importantly, have a relatively high stability under non-selective conditions. Nevertheless, the usefulness of the vectors described in EP 0 241 435 remains limited to research applications inasmuch as industrial-scale fermentations require a stability of at least 40 generations. Even the most efficacious vector (P3) described in EP 0 241 435 is lost by about 70% of the cells in a given population after only six generations in a non-selective medium (European patent application EP 0 241 435 A2, publ. 14.10.1987). Even though it is technically feasible to maintain a selection pressure in a large-scale fermentation, the use of selective medium often results in a considerably lower cell density, and requires the use of well defined strains, rendering this approach less attractive and more expensive. Furthermore, EP 0 241 435 does not contain single example of expression of a heterologous protein of commercial interest. In fact, the only "heterologous" gene shown to be expressed from vectors derived from pkD1 is the URA3 gene of the yeast S. cerevisiae. It hence remains to be proven that the introduction of a non-yeast gene, e.g. a gene of mammalian origin, into pkD1 and its overexpression in Kluyveromyces do not result in enhanced plasmid instability.
- object of the present invention is the production of new expression vectors capable of transforming yeasts of the genus Kluyveromyces and possessing stability characteristics markedly superior to those described in Patent Application EP 0 241 435. It will be shown that the new constructions described in the present invention are maintained at high copy numbers in 85-90% of the cells after 50 generations of growth in non-selective medium. It is thus possible to produce heterologous proteins from stable multicopy vectors using industrial strains of the genus Kluyveromyces which have been in use for many years on account of their optimal growth properties and at high cell densities.
- The present invention relates, in particular, to a method for the preparation of a specified protein, in which method a yeast of the genus Kluyveromyces transformed with an expression plasmid comprising:

 - genes A, B and C of plasmid pkD1,
 - the inverted repeats of plasmid pkD1 (IR).
 - the stability locus of pkD1.
 - the origin of replication of pkD1 and an expression cassette containing a DNA encoding the structural gene for said protein under the control of sequences permitting its expression in said yeast,
 - a selectable marker for the transformed yeast,
 - and, optionally, an origin of replication and a selectable marker for Escherichia coli.

- The high stability of the vectors described in the present invention has been achieved by exploiting fully the characteristics of plasmid pkD1. Vectors derived from pkD1 differ from all other known Kluyveromyces vectors in that they contain a specialized replication system responsible for stable plasmid maintenance at high copy number. In addition to an origin of replication this system comprises two inverted repeats, each 346 nucleotides in length and three open reading frames (genes A, B and C) which are integral parts of the plasmid (Chen, X.J. et al., Nucl. Acids Res. 14 (1986) 4471 - 4481). The retention of these replication and stability elements, genes A, B and C and inverted repeats (IR) results in vectors exhibiting a very high mitotic stability. By analogy with the most extensively studied system, that of the structurally related 2 u plasmid of S. cerevisiae, the proteins encoded by two of these genes (B and C) are probably involved in

plasmid partitioning during mitotic cell division, and might play a part in the negative regulation of gene A encoding a site-specific recombinase (FLP). Fletcher, A.B., *Yeast* 4 (1988) 27 - 40.

FLP-mediated recombination between the inverted repeats of 2 μ plasmids has been shown to switch the plasmid from a regular mode of replication (one doubling of 2 μ plasmids per cell division) to a rolling circle type mode of replication (copy number amplification to about 50 copies per cell; *ibid*). This change in the normal mode of replication is induced as soon as the plasmid copy number becomes too low to permit the production of sufficient quantities of gene B and C products which act as repressors of gene A encoding the FLP recombinase. By this mechanism, the copy number of 2 μ (and very probably of structurally similar plasmids such as pKD1) is maintained at high levels in an autoregulated manner and is independent of the presence of a selectable marker.

The vectors previously published in EP 0 241 435 either contain only a part of pKD1 (A15) or contain an interrupted gene A (P1 and P3 in which the Psi cloning site is located within the coding sequence of gene A (European patent application EP 0 410 1987), thereby destroying the autoregulated replication system which is one of the characteristics of the resident plasmid pKD1. In contrast, plasmid constructions derived from pKD1 described in the present invention respect the functional integrity of all the important open reading frames of pKD1. In consequence, the stability of the plasmids described below is considerably enhanced with respect to plasmids P1 and P3, and the copy number of the new vectors is maintained at a high level throughout the cell population.

In the present invention, the term HSA will be used to denote any serum albumin of human origin or any protein isolated from yeast cells and possessing the same amino acid sequence, tertiary structure and physicochemical properties as native HSA of human origin. Furthermore, HSA variants are understood to denote natural variants, as well as molecules having the same activity as HSA but from which, where appropriate, the domains not essential to the activity in question have been truncated.

The present invention relates to the creation by genetic engineering of new vectors enabling HSA to be produced in a readily purifiable form using yeast cells as a eukaryotic microbial host. A feature of the production method is the efficient excretion of mature, native HSA into the growth medium of said yeasts, thereby considerably facilitating the purification of the recombinant protein. Production of HSA is obtained by growing yeast transformed with a recombinant plasmid comprising a transcription and translation initiation region which functions efficiently in the said host and an open reading frame encoding HSA preceded by a secretion signal directing the recombinant protein to the secretory pathway of said yeasts.

According to the present invention, the new constructions are usually prepared in a stepwise manner employing the appropriate vectors until the elements essential to expression, secretion or plasmid maintenance have been combined to form a vector which can then be introduced into the defined host(s) so as to express and excrise the desired protein. The hosts employed are of eukaryotic origin, especially yeasts, more especially of the genera *Saccharomyces* or *Kluyveromyces* and preferably the species *Kluyveromyces marxianus* including all its varieties, especially *K. marxianus* var. *lactis*. Thus, according to the present invention, the constructions which are prepared and described are in the nature of examples in eukaryotic organisms of microbiological origin, but are preferably directed to *Kluyveromyces*.

The particular hosts to be employed will preferably be industrial strains which are stable, grow to high cell densities in appropriate media and have a high level of production.

Among the strains which can be transformed with pKD1-derived plasmids, strains of the species *K. wickerhamii*, *K. wallii* and, in particular, *K. marxianus* var. *bulgaricus* (*K. bulgaricus*), *K. marxianus* var. *granosiphilum* (*K. granosiphilum*), *K. marxianus* var. *marxianus* (*K. fragilis*) and *K. marxianus* var. *lactis* (*K. lactis*) should be mentioned more especially.

The HSA coding sequence may be obtained in a variety of ways, the simplest consisting in isolating messenger RNA from human liver and synthesizing copies thereof in the form of complementary DNA (cDNA). The cloned sequences may then be modified by different methods such as *in vitro* site-directed mutagenesis, primer elongation, restriction, insertion of adaptors or ligation with oligodeoxynucleotide linkers. The coding sequence may be adapted, for example, to the use of preferred codons in yeast in order to optimize the efficiency of protein synthesis (translation) in the said host.

At the N-terminus of the protein sequence, a signal peptide (pre-sequence) may be introduced so as to direct the nascent protein to the secretory pathway of the host cell. This pre-sequence may correspond to the natural N-terminal leader of the protein, in particular albumin, or it may be of another origin, e.g. it may be obtained from yeast genes such as those encoding the alpha pheromone or the killer toxin.

Furthermore, a pro-sequence encoding another peptide extension may be interposed between the secretory signal sequence and the coding sequence for mature albumin. This pro-sequence is normally joined to the coding sequence by a site for cleavage by a specific protease generally involving at least two

basic amino acids, preferably Lys-Arg or Arg-Arg.

The expression cassette will comprise a transcription and translation initiation region joined to the 5' terminus of the coding sequence, so as to direct and regulate the transcription and translation of said sequence. The choice of these promoter regions may vary according to the particular host used. These sequences are generally chosen from promoters derived from yeast genes. Of special interest are certain promoter and/or terminator regions derived from glycolytic genes of *Saccharomyces* or *Kluyveromyces* type yeast, such as the genes encoding phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GDP), endonuclease (ENO), alcohol dehydrogenases (ADH), or derived from other strongly expressed genes such as the lacZase (LACZ), the acid phosphatase (PHO5), and the like. These control regions may be modified, e.g. by *in vitro* mutagenesis, by introduction of additional control elements or synthetic sequences or by deletions. For example, transcription-regulating elements, such as the so-called UAS (upstream activating sequences) originating from another promoter, e.g. that of the *S. cerevisiae* *GAL10* or *K. lactis* *LAC4* gene, may be used to construct hybrid promoters which enable the growth phase of a yeast culture to be separated from the phase of expression of a heterologous gene, depending on the carbon source chosen. A transcription and translation termination region which is functional in the intended host will be positioned at the 3' end of the coding sequence.

The expression cassette thus constructed will be fused to one or more markers enabling the transformed host to be selected. Preferred yeast markers are dominant markers, i.e. markers conferring resistance to antibiotics such as gentamicin (G418) as is the case with the expression in said yeast of the *aph*(3'-I) gene (*aph*) of the bacterial transposon *Tn93*, or any other toxic compound such as copper ions, inasmuch as dominant markers may be used without special host requirements. These resistance genes will be placed under the control of appropriate transcription and translation signals in the host in question. It is also possible to use markers complementing auxotrophies such as the yeast genes *URA3* or *LEU2*.

The assembly consisting of the expression cassette and the selectable marker may be used either for transforming the host directly or it can be inserted into a replicative vector. In the case of a direct transformation, sequences homologous to regions present on a chromosome or a resident plasmid are fused to this assembly. Said sequences will be located on each side of the expression cassette so as to induce an insertion at the homologous site by *in vivo* recombination. The expression cassette can also be combined with a replication system which is functional in the host in question. A preferred replication system for *Kluyveromyces* is derived from the plasmid pKD1, initially isolated from *K. granosiphilum* (Falcone, C. et al., *Plasmid* 15 (1986) 248 - 252; Chen, X.J. et al., *Nucl. Acids Res.* 14 (1986) 4471 - 4481). A preferred replication system for *Saccharomyces* is derived from the yeast 2 μ plasmid. The expression plasmid can contain all or part of said replication systems or can combine elements derived from pKD1 as well as from the 2 μ plasmid. Preferred constructions are those which contain the entire sequence of the plasmid pKD1 when expression in *Kluyveromyces* is desired. More especially, preferred constructions are those in which the insertion site of the foreign sequences into pKD1 is localized in a 197 bp region lying between the *SacI* site (position 4714 of the B-form of pKD1; Chen, X.J. et al., *Nucl. Acids Res.* 14 (1986) 4471 - 4481) and *MspI* site (position 154 of the B form of pKD1; *ibid*), or at one of these two sites, or alternatively at the *SphI* site of plasmid pKD1.

For greater convenience the plasmid can be a shuttle vector: as such, it may be transferred to a bacterial host such as *E. coli* where it may be manipulated more readily than in yeast at each construction step. In this case, an origin of replication and a selectable marker functioning in the bacterial host are required. It is also possible to position restriction sites which are unique on the expression vector so that they flank the bacterial sequences. This enables the bacterial origin of replication to be eliminated by cleavage and the vector to be religated prior to transformation of the yeast cells, and this may result in a higher plasmid copy number and enhanced plasmid stability. Convenient sites such as 5'-GGCCNNNNNGGC-3' (Shu) or 5'-GGGGCGC-3' (Noll) can, for example, be used inasmuch as they are extremely rare in yeast and generally absent from an expression plasmid. These sites may be introduced into the vector by oligodeoxynucleotide-directed mutagenesis or by adding specific oligodeoxynucleotide linkers or adaptors.

Although the present description has been written with frequent reference to human albumin, the procedure using appropriate vectors derived from the plasmid pKD1 is applicable to albumins, interleukins, IP4, TIMP and other proteins capable of benefiting from expression/secretion in said yeasts.

Among coding sequences which may be expressed, there should be mentioned, in particular,

- H-1B
- pro-HSA
- Met-HSA
- pro-pro-PA

* Met-tPA, and
* TIMP.

Once construction of the expression vector has been completed, the latter will be introduced into the desired host. Various protocols for transformation have been described in the case of yeast (Sherman, F. et al., "Methods in Yeast Genetics", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1986). The transformants may then be grown in a suitable medium in order to produce the desired product. If the desired product is secreted, it may be purified in various ways from the culture supernatant. It may be coprecipitated, or extracted using affinity chromatography, electrophoresis or other conventional techniques. The secreted protein may be recovered from a batch culture or a continuous culture. The examples which follow are given without implied limitation, and show special embodiment of the invention and certain advantages.

DESCRIPTION OF THE FIGURES

Fig. 1: Construction of plasmid pXL276 containing the complete sequence encoding Met-HSA, obtained from three cDNA clones (see text) derived from poly(A)+mRNA isolated from human liver.
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Fig. 28: Mitotic stability of HSA expressing plasmids pYG19 and pYG23 under non-selective growth conditions: closed squares, strain MW98-8C transformed with plasmid pYG19; open squares, strain MW98-8C transformed with plasmid pYG23; open triangles, strain CBS63 transformed with plasmid pYG19.

Fig. 29: 8.5% SDS-polyacrylamide gel after staining with Coomassie Blue, demonstrating the expression and excretion of HSA from strain MW98-8C. Lanes 1-4: reference albumin extracted from human plasma (Sigma), spotted at increasing concentrations: 0.2 µg, 0.6 µg, 2 µg and 6 µg per lane. pYG19 (prepro-HSA), pYG23 (Met-HSA) and pYG25 (control vector without expression cassette); each lane corresponds to quantity of protein equivalent to 100 µl of the original culture. a) soluble fractions; b) insoluble fractions; c) culture supernatants.

Fig. 30: Immunoblotting of a 7.5% polyacrylamide gel blotted onto nitrocellulose as described in the text. Lane 1-5: reference albumin extracted from human plasma (Sigma) which has been dissolved in a YPD medium. Lane 1-5: reference albumin dissolved in YPD medium at 5% final concentration) as described. The samples of the HSA standards have hence been treated in the same manner as the culture supernatants. In both cases, the gel spots correspond to equivalent volumes. 1) precipitation experiments with a concentration of HSA of 100 mg/l; 2) 50 mg/l; 3) 25 mg/l; 4) 12.5 mg/l; 5) 6mg/l. pYG19 (prepro-HSA) and pYG23 (Met-HSA); each lane corresponds to a quantity of proteins equivalent to 20 µl of the original culture. a) culture supernatants; b) insoluble fractions; c) soluble fractions.

Fig. 31: 8.5% polyacrylamide gel after staining with Coomassie Blue, demonstrating the kinetics of excretion of HSA from strain MW98-8C.

Fig. 32: A, lanes a-e: reference HSA (Sigma) which has been dissolved in YPD medium at various concentrations and precipitated with TCA (5% final concentration) as described (see legend of Figure 21). a) concentration of HSA (Sigma) of 6 mg/l; b) 12.5 mg/l; c) 25 mg/l; d) 50 mg/l; e) 100 mg/l. pYG19-mediated secretion of HSA as a function of the age of the culture, 16-61 hours, as indicated. Each sample corresponds to the equivalent of 160 µl of culture supernatant.
 B, lanes a-e: reference HSA (Sigma), spotted at increasing concentrations: 0.4 µg, 0.6 µg, 0.8 µg, 1.0 µg and 1.2 µg per lane. pYG19: pYG19-mediated secretion of HSA as a function of the age of the culture, 72-240 hours, as indicated. Each sample corresponds to the equivalent of 25 µl of non-concentrated culture supernatant.

Fig. 33: Graphic representation of the kinetics of excretion of HSA from strain MW98-8C transformed with plasmid pYG19 and grown in an Erlenmeyer as described in the text. The concentration of albumin detected in the growth medium (mg/l) is shown as a function of the age of the culture (hours). The results of four independent experiments are shown.

B, growth curve of strain MW98-8C transformed with plasmid pYG19.

Fig. 34: Effect of carbon source on the efficiency of albumin secretion under control of the PGK or a PGK/LAC4 hybrid promoter: SDS-PAGE analysis of culture supernatants stained with Coomassie blue of K. lactis strain MW98-8C transformed with plasmids pYG19 and pYG44.

A, Supernatants (12.5 µl per lane) from shake-flask cultures grown for 21-1 h either in YPD (containing 2 % glucose) (lanes 1-3) or in YPD medium to which 2 % lactose has been added after 43 h of growth (lanes 4-6). Lanes a and b: HSA Sigma 0.5 and 1.0 µg, respectively; lanes 1 and 4: plasmid pYG44-5; lanes 2 and 5: plasmid pYG44-7; lanes 3 and 6: plasmid pYG19.

B, Supernatants (25 µl per lane) from shake-flask cultures grown for 11-3 h either in YPD (containing 2 % glucose) (lanes 1, 4 and 7), YPL (containing 2 % lactose) (lanes 3, 6 and 9) or in TYD medium to which 2 % lactose has been added after 43 h of growth (lanes 2, 5 and 8). Lanes a, b and c: HSA Sigma 0.25, 0.5 and 1.0 µg, respectively; lanes 7-9: plasmid pKan707 without expression cassette.

Fig. 34: Effect of promoter and signal sequence substitutions on the efficiency of albumin secretion: SDS-PAGE analysis of culture supernatants stained with Coomassie blue of K. lactis strain MW98-8C transformed with plasmids pYG51, pYG19 and pYG58.

A, Supernatants (25 µl per lane) from shake flask cultures grown for 115 h in YPD medium to which 2 % lactose has been added after 43 h of growth. Lanes a, b and c: HSA Sigma 0.2, 0.5 and 1.0 µg, respectively; lane 1: plasmid pYG51 (PHO5 promoter); lane 2: plasmid pYG19 (PGK promoter).

B, Supernatants (25 µl per lane) from shake flask cultures grown for 88 h (lanes 1-3) or 138 h (lanes 4-6) in YPD medium. Lanes a, b and c: HSA Sigma 0.25, 0.5 and 1.0 µg, respectively; lanes 1, 2, 4

and 5: plasmid pYG58 (killer toxin secretion signal, two independent transformants); lanes 3 and 6: plasmid pYG19 (albumin secretion signal).

Fig. 35: Effect of carbon source on the efficiency of albumin secretion under control of the LacC4 or the PGK promoter: SDS-PAGE analysis of culture supernatants stained with Coomassie blue of *K. lactic* strain CBS2359 transformed with plasmids pYG404 and pYG19.

Supernatants (25 μ l per lane) from shake flask cultures grown for 166 h either in YPD (containing 2 % glucose) (lanes 1 and 4), YPL (containing 2 % lactose) (lanes 3 and 6) or in YPD medium to which 2 % lactose has been added after 48 h of growth (lanes 2 and 5). Lanes a, b and c: HSA Sigma 0.25, 0.5 and 1.0 μ g, respectively; lanes 1-3: plasmid pYG404; lanes 4-6: plasmid pYG19.

Fig. 36: Efficiency of albumin secretion under control of an integrated versus a plasmid based PGK expression cassette: SDS-PAGE and immunoblot analysis of culture supernatants of *K. lactic* integrant strains MW98-8C::60-5 and MW98-8C::60-21, and of strain MW98-8C transformed with plasmid pYG19. Supernatants (25 μ l per lane) were sampled from shake flask cultures grown in YPD for 137 h.

A. PAGE-SDS. Lanes a, b, c and d: HSA Sigma 0.1, 0.25, 0.5 and 1.0 μ g, respectively; lane 1: strain MW98-8C transformed with plasmid pYG19; lanes 2-7: 6 independent integrant strains (MW98-8C::60-5#6, MW98-8C::60-21#7, MW98-8C::60-5#4, MW98-8C::60-5#8, MW98-8C::60-21#11, respectively; see section E.3.7).

B. Western blot analysis: same samples as shown in A (25 μ l of supernatant deposited per lane). Fig. 37: Efficiency of albumin secretion in various *Klebsiellales* strains transformed with plasmids pYG19 and pYG221B: SDS-PAGE analysis of concentrated culture supernatants stained with Coomassie blue. Cells were grown in YPD medium supplemented with G418 (200 μ g/ml) for 89 h. Supernatants were concentrated ten-fold by microfiltration using centricon 30 microfiltration units (Amicon). The volume of concentrated supernatant deposited per lane was standardized as a function of biomass production and values between 9 and 25 μ l.

Lanes a and b: HSA Sigma 0.5 and 1.0 μ g, respectively; lanes c and d: HSA Sigma subjected to microfiltration. A solution of HSA in YPD medium (10 mg/ml) was concentrated ten-fold by microfiltration. 25 and 2.5 μ l of the concentrated standard were deposited in lanes c and d, respectively. Lane 1: strain ATCC1645 (K. marxianus var. bulgaricus) transformed with plasmid pYG221B; lane 2: strain ATCC24178 (K. wickerhamii) transformed with plasmid pYG19; lane 3: strain ATCC12424 (K. marxianus var. marxianus var. lactis) transformed with plasmid pYG221B; lane 4: strain ATCC56500 (*K. wickerhamii*) transformed with plasmid pYG58; lane 5: strain ATCC66906 (K. marxianus var. drosophilaeum) transformed with plasmid pYG63; lane 6: strain CBS4574 (K. marxianus var. lactis) transformed with plasmid pYG19; lane 7: strain MW98-8C (K. marxianus var. lactis) transformed with plasmid pYG19; lane 8: strain MW98-8C (K. marxianus var. lactis) transformed with plasmid pYG221B.

Fig. 38: Electrophoretic techniques: S = reference HSA (Sigma), L = yeast albumin, 0.05 to 0.25 μ g. B - Isoelectric focusing (IPast gel, pH 4.5-6.0), staining with Coomassie Blue, spots = 1 μ g.

C. PAGE-SDS (7.5%) staining with Coomassie Blue. S = 0.2 - 1.0 μ g, L = 0.25-2.5 μ g.

Fig. 39: Ion exchange chromatography - Mono Q (Pharmacia). Bottom: yeast HSA 40 μ g; top: standard HSA 50 μ g.

Fig. 40: Reversed-phase chromatography = Nucleosil C4. a) standard HSA; b) yeast HSA.

Fig. 41: Tyrophan fluorescence emission spectrometry. Solid line: standard HSA; broken line: yeast HSA.

Fig. 42: Thermal stability at 75 °C. Solid line: standard HSA; broken line: yeast HSA. Fig. 43: Regions of the albumin recognized by the different monoclonal antibodies used for antigenic characterization.

Fig. 44: A,B,C: inhibition curves (see text) with respect to the nine monoclonal antibodies whose specificity is shown in Figure 43: percentage inhibition as a function of the albumin concentration (μ g/ml); open squares: standard HSA; filled squares: yeast HSA.

Fig. 45: Construction of plasmid pSPGK-L17 and indication of the nucleotide sequence of the synthetic EcoRI fragment used as a secretion signal. The nucleotide sequence surrounding the EcoRI cloning site of

plasmid pEPGK4 is also indicated.

Fig. 46: Construction of plasmid pSPGK-L17 and indication of the nucleotide sequence corresponding to the junction between the signal sequence and Met-II-1 β .

Fig. 47: Construction of plasmid pSPGK-L121.

Fig. 48: Construction of plasmid pSPGK-L131.

Fig. 49: Construction of plasmid pSPH104.

Fig. 50: Construction of plasmid pSPH10-L114.

Fig. 51: Construction of plasmid pSPH10-L123.

Fig. 52: Construction of plasmid pSPH10-L135.

Fig. 53: Stability curves of plasmids pCXXJ1, pSPGK-L131 and pSPH10-L135 in strain MW98-8C under non-selective conditions: filled squares, pCXXJ1; filled triangles, pSPH10-L135 (promoter not induced); open triangles, pSPH10-L135 (promoter induced); open squares, pSPGK-L131.

Fig. 54: Secretion of IL-1 β in strain MW98-8C: SDS-PAGE analysis of culture supernatants stained with Coomassie blue.

Each spotted sample corresponds to 0.2 ml of supernatant of *K. lactic* strain MW98-8C transformed with plasmid pKan707 (control vector, lane 1), pSPH10-L131 (lane 2) or pSPH10-L135 (lane 3); lane 4 corresponds to 1 μ g of Met-II-1 β produced in *E. coli* (Rhône-Poulenc Santé). The transformed cells were grown for three days in YPD medium supplemented with 200 μ g/ml of G418. The supernatants were concentrated by precipitation as described in the text.

Fig. 55: Restriction map of plasmids pXJ348 (Met-1 β) and pXJ459 (prepro-1 β A).

Fig. 56: Test of activity of the secreted 1 β A. 5 μ l of culture supernatant of *K. lactic* strain MW98-8C transformed with plasmid pYG3225B (prepro-1 β A, lane 1), pYG224B (Met-1 β , lane 2) or pKan707 (vector, lane 3) are spotted on a plate indicating 1 β A activity after 5 days of growth in selective YPD medium (200 μ g/ml of G418).

Fig. 57: Expression and secretion of TIMP in strain MW98-8C transformed with plasmid pKan707. 0.25 μ g of human TIMP (Rhône-Poulenc Santé, lane 1); intracellular recombinant TIMP (plasmid pYG226B), non-deglycosylated (lane 2) or after treatment with endoglycosidase H (lane 3); sample of *E. coli* recombinant TIMP (Rhône-Poulenc Santé, lane 4); intracellular fractions of strain MW98-8C transformed with the control vector pKan707; non-deglycosylated sample (lane 5) or after treatment with endoglycosidase H (lane 6); non-deglycosylated concentrated culture supernatants: strain MW98-8C transformed with plasmid pKan707 (lane 7) or pYG226B (lane 8); culture supernatants concentrated, then treated with endoglycosidase H; strain MW98-8C transformed with plasmid pKan707 (lane 9) or pYG226B (lane 10); 0.25 μ g of human TIMP Rhône-Poulenc Santé, lane 11).

EXAMPLES

General cloning techniques

Classical methods of molecular biology such as cesium chloride/bromide gradient centrifugation of plasmid DNA, restriction enzyme digestion, gel electrophoresis, electroelution of DNA fragments from agarose gels, transformation in *E. coli* and the like, are described in the literature (Maniatis, T. et al., "Molecular Cloning: a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982; Ausubel, F. M. et al. (eds.), "Current Protocols in Molecular Biology", John Wiley & Sons, New York, 1987).

The restriction enzymes were obtained from New England Biolabs (Biolabs), Bethesda Research Laboratories (BRL) or Amersham.

For ligation, DNA fragments are separated, according to size, on 0.7% agarose or 8% acrylamide gels, purified by electroelution, extracted with phenol, precipitated with ethanol and then incubated in a buffer comprising 50 mM Tris-HCl, 10 mM MgCl₂, 10 mM dithiothreitol, 2 mM ATP, pH 7.4, in the presence of phage T4 DNA ligase (Biolabs).

Where necessary, DNA fragments having 3' recessed termini are dephosphorylated by treatment with calf intestinal alkaline phosphatase (CIP, Pharmacia) at 37 °C for 30 minutes in the following buffer: 100 mM glycine, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.5. The same procedure is used for dephosphorylation of 5' recessed or blunt-ended termini, but the treatment is for 15 minutes at 37 °C and then 15 minutes at 56 °C. The enzyme is inactivated by heating the reaction mixture to 80 °C for 15 minutes in the presence of 1% SDS and 100 mM NaCl followed by extraction with phenol/chloroform and ethanol precipitation.

Filling in of the 3' recessed termini is performed with the Klenow fragment of *E. coli* DNA polymerase I (Biolabs). The reaction is performed at room temperature for 30 minutes in a buffer comprising 50 mM Tris-HCl, 0.4 mM dNTPs, 10 mM MgSO₄, 0.1 mM dithiothreitol, 50 μ g/ml BSA (bovine serum albumin), pH 7.2. Blunt-ending of 5' recessed termini is performed in the presence of phage T4 DNA polymerase (Biolabs) as

recommended by the manufacturer. Digestion of recessed ends is performed by limited treatment with S1 nuclease (BRL) as recommended by the manufacturer.

In vitro oligodeoxynucleotide-directed mutagenesis is performed according to the method developed by Taylor et al. (*Nucleic Acids Res.* 13(1985) 8749 - 8764), using the kit distributed by Amersham. Nucleotide sequencing is performed according to the dideoxy sequencing method (Sanger F. et al., *Proc. Natl. Acad. Sci. USA*, 74 (1977) 5463 - 5467). Enzymatic amplification of specific DNA fragments are performed by Polymerase-catalyzed Chain Reaction procedure (Mullis K.B. and Faloona F.A., *Meth. Enzyme*, 155 (1987) 355 - 356; Saiki R.K. et al., *Science* 230 (1985) 1350 - 1354) with a "DNA thermal cycler" (Perkin Elmer Censys) following the recommendations of the supplier.

Ligated DNA is used for transforming competent cells obtained from the following strains: *E. coli* MC1060 (*lacZ pro A*/*B*), supE, thi, his DS^r/TAD36, proA^r/*B*, lacZ^r M15. Plasmid DNA is purified from ampicillin- or tetracycline-resistant transformants as appropriate. Extraction of plasmid DNA is carried out according to the procedure described by Maniatis et al. (Maniatis, T. et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), which is derived from the alkaline lysis method described by Birnboim and Doly (Birnboim, H.C. and Doly, J., *Nucleic Acids Res.* 7 (1979) 1513 - 1523). For rapid plasmid analysis, bacteriolytes are prepared according to the method of Holmes and Quigley (Holmes, D.S. and Quigley, M., *Anal. Biochem.* 114 (1981) 193 - 197) and subjected to agarose gel electrophoresis analysis without prior purification. After endonuclease restriction analysis, recombinant plasmids exhibiting the desired structure are prepared on a larger scale according to the alkaline lysis procedure (Maniatis, T. et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor, N.Y., 1982) using 0.5 to 1 liter cultures, and purified by cesium chloride density centrifugation.

Transformation of *K. lactis* with foreign DNA and purification of plasmid DNA from *K. lactis* are described in the text.

Example 1: CLONING OF A CASSETTE CONTAINING THE PREPRO-HSA STRUCTURAL GENE

E.1.1 Isolation of a complementary DNA (cDNA) clone encoding HSA.

The construction of recombinant plasmids permitting the expression of HSA in *E. coli* has been described in detail in the previous Patent Application EP 0 198 745 (European patent application EP 198 745, pub. 22.10.1986). In summary, cDNA was obtained from poly(A)mRNA isolated from human liver according to the quanidine thiocyanate procedure (Chirgwin, J.M. et al., *Biochemistry* 18 (1979) 5294 pp.). DNA sequence analysis permitted the isolation of three clones (pT1B1, pAA3B and p63B, Figure 1) which contained overlapping fragments of the albumin structural gene and possessed common restriction sites within these overlaps. These sites were used in the reconstruction of a cDNA clone for HSA (Figure 1) which is complete except for its pre-pro-sequence.

E.1.2 Synthesis of the HSA prepro-sequence

In plasmid pXL276, the construction of which is described in detail in Patent Application EP 0 198 745, the coding sequence for mature HSA has been fused in frame to the ATG codon of the ribosome binding site (RBS) of the cII gene of bacteriophage lambda (Figure 2). This generated an NdeI site immediately upstream from the translation initiation codon of the HSA gene. pX276 is used for reconstituting the pre-pro-region of HSA, which is found to be truncated in the cDNA just upstream from the TagI site at amino acid "1". Reconstitution is achieved by inserting a DNA fragment corresponding to the HSA prepro-sequence in the form of four oligodeoxynucleotides 33-38 bases long (Sq32, Sq34, Sq35 and Sq38; Figure 2) and a 120 bp EcoRI-NdeI fragment derived from pUC18 carrying expression signals of *E. coli* genes between the EcoRI and AccI sites of plasmid pUC8. The TagI site is thus reconstituted at one end of the insert in this plasmid designated pXL260 (Figure 2). The small HindIII-TagI fragment carrying the RBS and the prepro-sequence upstream from the TagI site is ligated with the TagI-PstI fragment of the HSA cDNA clone (plasmid pB11, containing the 5' end of HSA) between the HindIII and PstI sites of pUC8 to give plasmid pXL256 (Figure 3). Plasmid pXL322 is constructed by ligating the HindIII-PvuII fragment of pXL299, carrying the RBS and the prepro-HSA sequence up to the PvuII site, with the large PvuII-EcoRI fragment of pXL276, bearing the replicon and the 3' end of the HSA coding sequence, and with the EcoRI-HindIII

fragment of pXL276 containing the promoter (Figure 4).

E.1.3 Creation of a HindIII site upstream from the HSA translation initiation codon

In order to obtain a prepro-HSA cassette capable of being readily integrated in expression vectors, the NdeI site of plasmid pXL322, described above, is changed into a HindIII site by oligodeoxynucleotide-directed mutagenesis. To this end, the HindIII-BglII fragment of pXL322 containing the 5' end of the prepro-HSA gene is subcloned into M13mp18 and mutagenized by hybridizing the synthetic oligodeoxynucleotide 5'-ATCTAAGGAATAC AACGCTTATGAA GTGGGT-3' to the single-stranded template (the underlined and bold-type sequences represent the HindIII site and the translation initiation site, respectively). Plasmid pXL859 was thus obtained (Figure 5), of which the nucleotide sequence of the mutagenized region was verified by using the dideoxy sequencing method. The sequence encoding the complete prepro-HSA is reconstituted by inserting the HindIII-PvuII fragment of the mutagenized phage and the PvuII-HindIII fragment of pXL322, containing the 3' end of the HSA structural gene, into the HindIII site of pUC8 to give plasmid pXL869 (Figure 6). This plasmid hence contains a 1.87 kb HindIII fragment containing the complete prepro-HSA structural gene as well as an untranslated 61 bp region at its 3' end. The complete sequence of the HindIII fragment as well as the amino acid sequence of the recombinant HSA, are depicted in Figure 7. A HindIII cassette containing the Met-HSA structural gene without any secretion signal is constructed by following the same procedure except that an M13 derivative of plasmid pXL276 is mutagenized using the synthetic oligodeoxynucleotide 5'-ATCTAAAGGAAATACAAGGCTTATGAT GCACAAAG-J. Reconstitution of the complete Met-HSA coding sequence in pUC8 gives plasmid pXL868, obtained in a similar manner to plasmid pXL869.

Example 2: CONSTRUCTION OF YEAST CLONING VECTORS

E.2.1 Isolation and purification of plasmid pKD1

Plasmid pKD1 may be purified from a late logarithmic phase culture of *K. drosophilaeum* strain UCD 51-130 (U.C.D. collection, University of California, Davies, CA 95616) according to the following procedure which is derived from that described by Fleier et al. (*Mol. Cell. Biol.* 7 (1987) 1180 - 1192). A 1 liter culture in YPD medium (1% yeast extract, 2% Bacto-peptone (Dico), 2% glucose) is centrifuged, washed and resuspended in a 1.2 M sorbitol solution, and the cells are converted to spheroplasts in the presence of zymolase (300 µg/ml), 25 mM EDTA, 10 mM phosphate and β-mercaptoethanol (1 µg/ml). After washing in 1.2 M sorbitol solution, the spheroplasts (corresponding to 250 ml of the original culture per tube) are resuspended in 2.5 ml of 1.2 M sorbitol and the same volume of buffer comprising 25 mM Tris-HCl, 50 mM glucose and 10 mM EDTA, pH 8.0, is added. The subsequent steps correspond to the alkaline lysis procedure already described (Maniatis, T. et al., "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1982), except for the precipitation of the DNA which is carried out at 23 °C for 15 minutes by adding 14 ml of isopropanol. The material obtained is treated with RNase (50 µg/ml) at 37 °C for 20 minutes and then with proteinase K (150 µg/ml) in a solution comprising 0.5 M NaCl, 0.5% sarcosyl and 25 mM EDTA for 1 hour at 60 °C. After centrifugation in an Eppendorf microcentrifuge, the supernatants are precipitated with ethanol for 10 minutes at -70 °C. The pellets are then dissolved and the DNA is purified by CsCl density gradient centrifugation in the presence of ethidium bromide.

E.2.2 Construction of plasmid pCXJ1

The construction intermediate pUC-URA3 (Figure 8) consists of a 1.1 kb HindIII fragment containing the URA3 gene of *S. cerevisiae* inserted into the unique NdeI site of plasmid pUC19 (Yanisch-Perron, C. et al., *Gene* 33 (1985) 103 - 119). The HindIII fragment is derived from plasmid pG63 (Gerbaud, C. et al., *Curr. Genet.* 3 (1981) 173 - 180) which has been digested with HindIII and then treated with the Klenow fragment of *E. coli* DNA polymerase I to generate blunt-ended termini. The 1.1 kb fragment containing the URA3 gene is purified and then inserted into the plasmid pUC19, itself cut with NdeI and treated with the Klenow fragment of *E. coli* DNA polymerase I. Plasmid pUC-URA3 hence contains: an origin of replication for maintenance of the plasmid in *E. coli*, the ampicillin resistance marker for transformations in *E. coli*, the

K_l lacZ gene containing a polylinker (EcoRI, SacI, BamHI, XbaI, Sall, SphI, HindIII as unique sites) and the URA3 gene of S. cerevisiae serving as a selectable marker in uraA mutants of K. lactis.

5 AaIII site of pUC-URA3. This construction has been obtained by linearizing pKD1 at the EcoRI site and then treating this plasmid with the Klenow fragment of E. coli DNA polymerase I. This DNA fragment is ligated with pUC-URA3 previously cut with AaIII and treated with T4 DNA polymerase. Ligation of these two fragments permits reconstitution of the EcoRI restriction sites. The insertion of the DNA of pUC-URA3 at the EcoRI site of the plasmid pKD1 does not inactivate any gene required for maintaining plasmid stability and copy number (the EcoRI site being located 205 nucleotides (nt) upstream from the ATG codon of gene B; Chen, X.J. et al., Nucl. Acids Res. 14 (1986) 4471 - 4481.). In consequence, plasmid pCXJ1, which transforms K. lactis uraA cit⁰ cells at high frequency, is amplified to about 70-100 copies per cell and stably maintained even in the absence of selection pressure. Due to the origin of replication constructed by pUC-URA3, plasmid pCXJ1 can also replicate in E. coli, thereby facilitating the plasmid construction and purification steps. The unique sites of plasmid pCXJ1 which can be used to insert foreign DNA and the HindIII and Sall sites from the pUC19 polylinker.

E.2.3 Construction of a fusion between the K. lactis P_{ur} promoter and the 3'aminoglycoside phosphotransferase gene of Tr903

20 The use of pCXJ1 as a vector for the transformation of K. lactis and other Kluyveromyces species remains limited to strains carrying the chromosomal uraA mutation as an auxotrophic marker. In order to be able to transform industrial wild-type strains of Kluyveromyces, the 3'-aminoglycoside phosphotransferase (aph) gene of the bacterial transposon Tr903 was chosen as a dominant antibiotic resistance marker which was inserted into pCXJ1. The aph gene confers resistance to kanamycin in E. coli and if expressed in wild-type yeast strains confers resistance to the antibiotic G418 (geneticin), which is a potent inhibitor of cell growth (Jimenez, A. and Davis, J., Nature 287 (1980) 869-871). To permit a sufficiently strong expression of the aph gene in K. lactis, the bacterial transcription signals of the aph gene are replaced by the ORF1 promoter (P_{ur}) isolated from the killer plasmid k1 of K. lactis.

25 The construction of the P_{ur}-aphfusion is performed in several steps (Figures 10 to 12). In the first place, a 1.5 kb Scal-PstI fragment of plasmid k1 is subcloned between the unique Scal and PstI sites of pBR322; the ampicillin-sensitive and tetracycline-resistant recombinant plasmid is designated p_{k1}-PSI535-8 (Figure 10). The 1.5 kb Scal-PstI subcloned fragment is derived from one extremity of the linear killer plasmid; it contains the 5' half of ORF1 carried by plasmid k1 and about 220 base pairs upstream (Sor, F. and Fukuhara, H., Curr. Genet. 9 (1985) 147 - 155). Since the Scal site is located only 22 base pairs from the left-hand extremity of k1 (Figure 10), the 1.5 kb Scal-PstI fragment probably contains the entire promoter region of ORF1 (P_{ur}). Digestion of p_{k1}-PSI535-8 with DraI yields a 268 bp fragment containing 17 bp derived from pBR322 at one of its extremities (close to Scal) and the first 11 codons of ORF1 at the other extremity. After treatment with the Klenow fragment of E. coli DNA polymerase I, the purified fragment is inserted into the unique XbaI site of plasmid pUC-k_{kan}1 (Figure 11). The latter plasmid was obtained by inserting a 1.25 kb EcoRI fragment containing the aph genes derived from Tr903 (Kanamycin Resistance Gene Block™, Pharmacia) into the unique EcoRI site of pUC19. Plasmid pUC-k_{kan}2 is obtained by performing a digestion of pUC-k_{kan}1 with XbaI, followed by a brief digestion with S1 nuclease, rendering the termini blunt-ended, followed by ligation with the DdeI fragment of p_{k1}-PSI535-8 rendered blunt-ended with the Klenow fragment of E. coli DNA polymerase I (Figure 11). This construction enables an intramolecular recombination between the first 11 amino acids of the ORF1 gene of the linear plasmid k1 and the 5' truncated end of the aph gene of Tr903. In fact, the junction between ORF1 and aph restores the twelve codons of aph (AGG) so that the first eleven amino acids of aph have been replaced by the first eleven amino acids of ORF1. The complete sequence of the ORF1 promoter used for the P_{ur}-aph fusion together with the beginning of the structural gene encoding aph is shown in Figure 12.

E.2.4 Construction of plasmid pKan707

55 Plasmid pKan707 (Figure 13) is a derivative of plasmid pCXJ1 described above. It is constructed by cutting plasmid pCXJ1 with HindIII and then treating with Klenow fragment of E. coli DNA polymerase I. The linearized plasmid with blunt-ended termini is then ligated with a 1.2 kb Scal-HincII fragment derived from plasmid pUC-Kan202 carrying the P_{ur}-aph fusion (Figure 13). Digestion of pUC-Kan202 with Scal and

HincII yields a blunt-ended kanamycin resistance cassette no longer containing the original bacterial promoter. The plasmid obtained, pKan707, confers resistance to very high levels of G418 (> 2.5 g/l) in K. lactis strains. As in the case of pCXJ1, plasmid pKan707 can be introduced into K. lactis cit⁰ strains at high frequency, amplified to 70 - 100 copies per cell and stably maintained in the absence of selection pressure (Figure 14). The great sensitivity to G418 of most Kluyveromyces strains combined with the presence of an efficient dominant marker permitting transformation of industrial strains makes pKan707 a very useful cloning vector for yeasts of the genus Kluyveromyces.

E.3 CONSTRUCTION OF EXPRESSION VECTORS CONTAINING THE EXPRESSION AND/OR SECRETION CASSETTES FOR ALBUMIN IN YEAST

E.3.1 Construction of expression cassettes for Meth-HSA and prepro-HSA under the control of the PGK promoter or S. cerevisiae

Plasmid pYG12 (Figure 15) contains a 1.8 kb Sall-BamII restriction fragment consisting of the promoter and terminator regions of the PGK gene of S. cerevisiae. This fragment is derived from a 3.0 kb HindIII genomic fragment from which a 1.2 kb fragment corresponding to the structural gene has been deleted and comprising a region between the translation initiation ATG and the BglII site localized 30 codons upstream from the TAA codon specifying the termination of translation (Mellor, J. et al., Gene 24 (1983) 1 - 14). The HindIII sites flanking the 1.8 kb fragment which are thereby obtained are then destroyed and replaced by a Sall and BamII site, respectively upstream from the promoter region and downstream from the PGK transcription terminator. A HindIII site is then introduced at the junction between promoter and terminator regions; since the site is unique, it enables heterologous genes to be readily introduced. The nucleotide sequence of this region is shown in Figure 15. The 1.8 kb HindIII fragment derived from plasmid p_{YG}88 (see E.1.3) and encoding Meth-HSA is then introduced into the HindIII site of plasmid pYG12 to give the recombinant plasmid pYG10. In a similar manner, plasmid pYG11 is generated by insertion into plasmid pYG12 of a HindIII fragment derived from plasmid p_{YG}89 (see E.1.3) and encoding prepro-HSA (Figure 15). In consequence, two Sall-BamII expression cassettes about 3.6 and 3.7 kb in size are thus constructed, comprising the PGK gene promoter of S. cerevisiae (P_{PGK}) followed by the gene encoding either Meth-HSA or prepro-HSA, and finally the region corresponding to the transcription terminator of the PGK gene (T_{PGK}), a region including the polyadenylation site of the mRNA.

E.3.2 Construction of expression plasmids p_{YG}19, p_{YG}23 and p_{YG}221.

With the object of introducing the expression cassettes described above into the vector pKan707, the 3.8 and 3.7 kb Sall-BamII fragments derived from plasmids pYG10 and pYG11 are, in a first stage, subcloned into the corresponding sites of plasmid pIC-20R (Marsh, L. et al., Gene 32 (1984) 481 - 485); to give in this manner the plasmid constructions p_{YG}19 (prepro-HSA) (Figure 16) and p_{YG}221 (Meth-HSA). These construction intermediates give rise to P_{PGK}-Meth-HSA/T_{PGK} and P_{PGK}-prepro-HSA/T_{PGK} expression cassettes in the form of Sall-SacI restriction fragments which can be directly inserted into the corresponding sites of pKan707 (Figure 17). Digestion of the latter with the enzymes Sall and SacI leads to deletion of the URA3 marker as well as that of a 35 bp fragment located upstream from gene B of pKD1. The constructions thereby obtained, designated p_{YG}19 (prepro-HSA) (Figure 18) and p_{YG}23 (Meth-HSA) comprise the P_{PGK}-HSA/T_{PGK} expression cassettes, the virtually complete sequence of K. drosophilum plasmid p₀₁. The sequences permitting the autonomous replication of E. coli as the genes encoding β -lactamase and 3'-aminoglycoside phosphotransferase (under the control of the P_{ur} promoter), enabling, respectively, E. coli to be selected in the presence of ampicillin and K. lactis in the presence of G418.

55 p_{YG}19, p_{YG}23 and p_{YG}221 are shown in Figure 19A, plasmid p_{YG}208 is derived from plasmid pKan707 by inserting a BamII/Sall adapter (5'-GATCCGTCCGACG-3') downstream from the PGK terminator. The HindIII fragment encoding prepro-HSA is purified by electroelution from plasmid p_{YL}89 (prepro-HSA), and cloned in the correct orientation into the HindIII site of vector p_{YG}208 to generate plasmid p_{YG}210; the Sall expression cassette thereby obtained (PGK promoter/prepro-HSA/PGK terminator) is purified by electroelution and cloned into the Sall site of plasmid pKan707 to generate expression plasmids p_{YG}221A and p_{YG}221B (Figure 19B), which only differ from one another in the relative orientation of the Sall expression cassette with respect to the vector

pkn707. In plasmid PYG221B, the orientation of the Sall expression cassette relative to the Km' gene coding for resistance to G418 is identical to that of the Sall-SacI expression cassette carried by plasmid PYG19.

E.3.3 Construction of a PGK/LAC hybrid promoter with optimized ATG context

In order to obtain an inducible derivative of the PGK promoter present in expression plasmid PYG19, the UAS of the PGK promoter was replaced by the UAS derived from the LAC4 promoter of *K. lactis*. (Braunig, K.D. et al., Nucl. Acid Res. 12 (1984) 2327 - 2341). To this end the Sall-HindIII fragment of PYG12, containing the PGK promoter was cloned into bacteriophage M13mp18 giving rise to construction PYG14 (Figure 20). Using the protocol described above, two NotI sites were then introduced into the PGK promoter region via site directed mutagenesis, generating construction PYG28 (Figure 20). The synthetic oligonucleotides used for this purpose were:

15 5'-CAAACTCGTGAAGCCGCTAGAACGCTGGCTTATG-3' (Sq417) and
5'-GG TAATTCTCTTCGATAAGCQCCACGCTGGCTTATG-3' (Sq418).

Thereby a total of four basepair changes (underlined) were introduced at positions -397, -399, -536, and -541 (with respect to the ATG initiator codon defined as +1; Figure 21). The two NotI sites (bold letters) obtained in this way flank the region of the PGK promoter which has previously been described to contain two functionally distinct domains (A and M in Figure 21 A) of the UAS_{LAC} (Stanway, C. et al., Nucl. Acids Res. 15 (1987) 6855 - 6873). Digestion of this PGK derivative with the NotI allows deletion of the UAS_{LAC} and its subsequent replacement by a synthetic fragment containing the UAS_{LAC} flanked by NotI sites (Figure 22), cloned in either orientation with regard to the PGK promoter. The UAS_{LAC} fragment was obtained by hybridization and subsequent ligation of the following four oligodeoxynucleotides:

25 Sq426, upper strand, fragment 1:
(-448) 5'-GGC CGC TGT GAA AAG TGT AGC GGA AAT ATG TGG TCC GAG CAA CAG CGT CTT TTG CTA GTAA

Sq427, upper strand, fragment 2:
(-398) 5'-TCG GTT ACT TGG ACA TTG GTAA TTT GGA CTT TGT TGC TAC ACC ATT CAC TAC TCT TTG

Sq428, lower strand, fragment 1:
(-319) 5'-GGC CGC TCA CAC TCG ACT TCA AGT AGT GAA AAC AAA GTC CAA ATA

Sq429, lower strand, fragment 2:
(-382) 5'-AAC CGA CCG CAC TAC TAG AAA AAG AGC CTG TTG CTC GGA CCA CAT ATT TCC GCT

30 ACA CCT TTC AGA AGC-3' (-319)

35 Alacovic, M. et al. (eds.) 551 - 560; Leonhardt, J.M. et al., Mol. Cell. Biol. 7 (1987) 4369 - 4376). Letters printed in bold face represent nucleotides which are part of the two NotI sites flanking the synthetic UAS of the LAC4 gene; the junction sequence of Sq27 complementary to Sq25 is underlined.

The region spanning positions -328 to -435 has been shown to harbour the first of three UAS elements found in the LAC4 promoter (Breuning, K.D. et al., 5th Internat. Sympos. Genet. Indust. Microorgan., (1986) 40 Alacovic, M. et al. (eds.), 551 - 560; Leonhardt, J.M. et al., Mol. Cell. Biol. 7 (1987) 4369 - 4376). Letters printed in bold face represent nucleotides which are part of the two NotI sites flanking the synthetic UAS of the LAC4 gene; the junction sequence of Sq27 complementary to Sq25 is underlined.

Since the nucleotide sequence near the initiator ATG of highly expressed eukaryotic genes is thought to influence the affinity of translation initiation (Kozak, M., Microbiol. Rev. 47 (1983) 1 - 45; Hamilton, R. et al., Nucl. Acid Res. 15 (1987) 3381 - 3383), the ATG context of the PGK promoter was modified as follows: via site directed mutagenesis an additional HindIII restriction site (bold face) was introduced at position -25 of the PGK promoter carried on PYG28. Thus generating plasmid pYG29 (Figure 20). The synthetic oligodeoxynucleotide used for this experiment was 5'-TATTTTGTTAAAGCTTGTATAATTCTTCC-3'.

50 The UAS_{LAC} fragment described above was then cloned into the NotI sites of PYG29 resulting in plasmids pYG3-1 and pYG3-2 (Figure 22). The PGK/LAC4 hybrid promoter regions of the latter plasmids were purified as Sall-HindIII fragments and ligated to a synthetic HindIII/BstEII adaptor composed of the following two complementary oligodeoxynucleotides: (i) 5'-AGC TTT ACA ACA AAT ATA AAA ACA ATG AAG TGG-3' (Sq451) and (ii) 5'-GT TAC CCA CTT CAT TGT TTT TAT TGT TGT AA-3' (Sq452) (the prepro-HSA initiator codon is represented in bold letters). This adaptor reconstitutes the 22 bp immediately upstream of the wild-type PGK promoter (Figure 21 B) and comprises the first four codons of the prepro-HSA structural gene up to a naturally occurring BstEII site. The Sall-BstEII fragments obtained in this manner (carrying the PGKLAC4 hybrid promoter with an optimized initiator ATG context) were then used to replace

the corresponding restriction fragment (harbouring the non-mutagenized PGK promoter) in construction PYG18 (c.f. Figure 16). Following this strategy two construction intermediates were obtained which only differ in the orientation of the UAS_{LAC} with regard to the PGK promoter. These new constructs were used to generate Sall-SacI expression cassettes which were cloned into the corresponding restriction sites of pkn707 (c.f. Figure 13), generating plasmids pYG44-5 and pYG44-7 (Figure 23). These plasmids are isogenic except that the synthetic NotI fragment which harbours the UAS_{LAC} is in the wild-type orientation for pYG44-5 and in the opposite orientation for plasmid pYG44-7.

E.3.4 Construction of a vector for preproHSA secretion under control of the PHOS promoter of *S. cerevisiae*

Vector pEPHO (Figure 24) contains a 0.34 kb BamHI-PstI restriction fragment harbouring the promoter and terminator region of the acid phosphatase gene (PHO5) of *S. cerevisiae*. This fragment is derived from a 2.03 kb genomic BamHI fragment (pHO5) of *S. cerevisiae*. This fragment is derived from the PHO5 structural gene. This fragment comprises positions 3 (with respect to the ATG initiator codon +1) and +1109 (the Sau3A site located upstream of the TAG terminator codon). A polylinker EcoRI-SmaI-BamHI was inserted at the junction between promoter and terminator and can be used as cloning site for the introduction of a heterologous gene. The junction between the PHO5 promoter and this polylinker is given below the polylinker of bacteriophage M13mp9 and the resulting single-stranded 5'-AAATTTCGCAATTAGGAATTCCGGCGCTTC-3'.

In order to obtain a construct in which the HSA gene is expressed under control of the PHO5 promoter and the non-translated leader region of the PGK gene, a HindIII site was introduced at position -20 of the PHO5 promoter fragment derived from plasmid pEPHO. To this end, the 0.83 kb Sall-EcoRI fragment of pEPHO was subcloned into the polylinker of bacteriophage M13mp9 and the resulting single-stranded template (pYG5F3) was then mutagenized using the oligodeoxynucleotide 5'-CCCTAATCTTCGATAAAGCTTGCCTCATTTGGCTG-3' (Sq487); the HindIII site introduced is represented in bold characters) as mutagenic primer, resulting in plasmid pYRF4 (Figure 24).

To facilitate the construction of a variety of expression cassettes containing different promoters and/or polylinkers was destroyed by treatment of the cohesive ends with the large fragment of polymerase I (Khanlo) of *E. coli*. This new cloning vector (pIC-20RΔH) was then used to introduce the Sall-BamHI fragment of plasmid pYG12 (c.f. Figure 15) containing the promoter and terminator regions of the PGK gene, thus generating plasmid pYG61. By using promoter modules as HindIII-HindIII fragments and terminator modules as HindIII-BamHI fragments, expression cassettes can be generated with any combination of transcriptional initiation and termination signals into which the structural gene to be expressed has been cloned as a HindIII restriction fragment. Digestion of these construction intermediates with the enzymes Sall and SacI allows subsequent introduction of the new expression cassettes into the corresponding sites of yeast cloning vector pKan707 (c.f. Figures 17 and 18).

Following this basic strategy, the Sall-HindIII PGK promoter fragment of plasmid pYG61 was replaced by the Sall-HindIII fragment of pYRF4. Treatment of the PHOS promoter, insertion of the prepro-HSA structural gene isolated from plasmid pYG44-5 as HindIII fragment (c.f. Figure 23) and transfer of the resulting expression cassette as SalI-SacI fragment into plasmid pKan707 give rise to secretion vector pYG51 (Figure 23). This plasmid thus contains the PHO5 promoter of *S. cerevisiae* up to position -17 (including all transcription initiation sites (Rudolph, H. and Hinzen, A., Proc. Natl. Acad. Sci. USA 84 (1987) 1340 - 1344), Figure 25), followed by 21 bp of the untranslated PGK leader sequence, the prepro-HSA structural gene, and finally the PGK terminator.

E.3.5 Construction of a vector for HSA secretion under control of the LAC4 promoter of *K. lactis*

Using the polymerase-catalyzed chain reaction (PCR) technique, the LAC4 promoter of *K. lactis* was amplified from yeast genomic DNA as a Sall-HindIII restriction fragment. Total genomic DNA isolated from strain CBS2359 served as template and the following synthetic oligodeoxynucleotides were used as primers for the Tag-polymerase reaction (the Sall and HindIII sites introduced upstream and downstream, respectively, of the LAC4 promoter of *K. lactis* are represented in bold letters):

21 5'-CCCGTCGACATGAGTAGACAAAGACAGGGAGGGC-3
22 5'-AAAGCTTATCTTTCAGTTCTCGATGAGTATGTGTT-3

The design of these primers was based on the LAC4 sequence previously published by Leonardo J.M. et al. (*Mol. Cell. Biol.* 7 (1987) 4369 - 4376). The Sall-HindIII restriction fragment obtained in this manner contains the LAC4 promoter region spanning positions -1798 to -1 (the ATG initiator codon being defined as +1). The amplified material was digested with the enzymes Sall and HindIII, cloned into plasmid pYG61 (c.f. section E.3.4), and its nucleotide sequence was confirmed using the di/deoxy chain termination method. Following the general scheme described in the previous section a pkan707 derivative was obtained, designated pYG404 (Figure 23), in which the prepro-HSA gene is expressed under the control of the lactose galactose inducible LAC4 promoter of K. lactic. The sequence immediately upstream of the HSA initiator codon in this specific construction is derived from plasmid pYG445 (fragment HindIII) and therefore contains part of the untranslated leader of the PGK gene (c.f. section E.3.3).

E.3.6 Construction of a vector for HSA secretion under control of the PGK promoter of S. cerevisiae and the killer toxin secretion signal of K. lactic

Using two pairs of complementary synthetic oligodeoxynucleotides we reconstituted a HindIII-DraI fragment which contains the following elements: (i) the ATG proximal 21 bp of the PGK promoter, (ii) the secretion signal sequence ("pre"-region) of the killer toxin gene from plasmid K1 of K. lactic (Stark M.J.R. and Boyd A., *EMBO J.* 5 (1986) 1995 - 2002), and (iii) the first 17 amino acids of the pro-HSA gene up to the N-terminal DraI site, fused in frame to the killer toxin pre-region. The four oligodeoxynucleotides used for this construction were as follows:

Sq900, upper strand, fragment 1:
5'-AGCTTACCTTACACAATATAAACAAATGATAATTTACATATTTCGTTGCATGGTTGCATGGTT-3'

Sq98, upper strand, fragment 2:
5'-ATTCTGTTCAAGGGTAGGGGTGTTGCTGAGATGCACACAAAGGTGGGGTGCTCATGGTT-3'

Sq98, lower strand, fragment 1:
5'-AAAACCAAATATGTAATTCAATTGTTTATATTGTTGAAGCTA-3'

Sq97, lower strand, fragment 2:
5'-AAACCGTGACGACACTCTCTGTCGACGAAACACCCCTACCTGAAACGAATGACAG-

C-3

Letters printed in bold face represent nucleotides which are part of the HindIII and DraI sites used in this experiment; the junction sequence of Sq98 complementary to Sq966 and Sq98 is the underlined. After phosphorylation of oligodeoxynucleotides Sq966 and Sq98, a double stranded HindIII-DraI fragment was generated according to the scheme depicted in Figure 26. This 130 bp fragment was purified by gel electrophoresis and ligated to a 1.04 kb DraI-XbaI fragment containing part of the HSA structural gene isolated from plasmid pYL869 (c.f. Figure 6). The HindIII-XbaI fragment obtained in this manner was then cloned into bacteriophage M13mp10, generating construction pYG56 (Figure 26). The correct nucleotide sequence of this intermediate was confirmed by dideoxy sequencing.

The entire propro-HSA structural gene can then be reconstituted by ligation of the following three elements: (i) the 1.17 kb HindIII-XbaI fragment derived from the pYG56 construct just described, (ii) the XbaI-BamHI fragment of pYG58 (c.f. Figure 16) containing the carboxy-terminal half of the HSA gene as well as the PGK terminator region, and (iii) a BamHI-HindIII fragment of plasmid pYG62. This latter plasmid is a derivative of construction pYG61 (c.f. section E.3.4) in which the Sall-HindIII fragment carrying the PGK promoter has been replaced by the equivalent restriction fragment derived from plasmid pYG58 (c.f. Figure 45). The BamHI-HindIII fragment of plasmid pYG62 used in this construction thus contained the E. coli origin of replication, the ampicillin resistance gene, and the PGK promoter with a HindIII site introduced at position -25 (c.f. section E.3.4). The resulting pIC derivative was designated pYG57. This latter plasmid was used as source of a SalI-SacI fragment which was cloned into the corresponding sites of yeast cloning vector pKan707 giving rise to expression vector pYG58 (Figure 23).

E.3.7 Construction of an integrative vector for directing a PGK/preproHSA secretion cassette to the RAG2 locus of K. lactic

Plasmid p31/RAG2:URA3 (Figure 27), kindly provided by Dr. M. Wesołowski-Louvel (Institut Curie, Paris, France), contains a 2.3 kb genomic NheI fragment harbouring the promoter and N-terminal region of the RAG2 structural gene of K. lactic which most likely encodes a phosphoglucomutase isomerase (Wesołowski-Louvel M. et al., *Nucl. Acid Res.* 16 (1988) 8714). Plasmid p31RAG2:URA3 was constructed by insertion of

a 1.6 kb EcoRI fragment derived from plasmid pCXJ1 (c.f. Figure 9), harbouring the URA3 gene of S. cerevisiae, into the EcoRI site of the RAG2 promoter. The 3.9 kb NheI fragment obtained in this way was then cloned into the unique NheI site of plasmid pBR322.

By cutting plasmid pYG18 (Figure 16) with the restriction enzymes EcoRV and SmaI a 3.7 kb fragment was obtained containing the same PGK prepro-HSA expression cassette as the one used in yeast secretion vector pYG19 (Figure 18). Insertion of this cassette into the unique SmaI site of plasmid p31RAG2:URA3 (located 3' of the URA3 marker gene) generated constructions pYG60-1 and pYG60-5 (Figure 27) which only differ from each other with respect to the orientation of the prepro-HSA expression cassette.

By cutting plasmids pYG60-1 and pYG60-5 with the enzyme NheI a 7.7 kb fragment was generated containing the HSA expression cassette and the URA3 marker gene flanked by RAG2 derived sequences. These latter sequences served to target the integration of the entire fragment to the RAG2 locus of K. lactic strain MW98-8C via homologous recombination (Rothstein R.J., *Methods in Enzymol.* 10 (1983) 202 - 211). Integration at the RAG2 locus was confirmed by Southern analysis for three out of six integrants tested (MW98-8C::60-5 integrant clone #8 and MW98-8C::60-21 integrants clones #7 and 9). The remaining three integrants were found to carry one (MW98-8C::60-5 integrant clone #8 and MW98-8C::60-21 integrant clone #11) or two (MW98-8C::60-5 integrant clone #4) HSA expression cassettes integrated at loci other than RAG2. All six of the confirmed integrants were tested for efficiency of HSA secretion.

E.4 TRANSFORMATION OF KLUVEROMYCES SPECIES WITH PLASMIDS EXPRESSING HSA

E.4.1 Transformation procedures

Transformants of K. lactic strain MW98-8C (lac ura arg lys K^r pkD1) were obtained either by the spheroplast formation technique initially described by Hinman et al. (Hinman, A. et al., *Proc. Natl. Acad. Sci. USA* 75 (1978) 1929 - 1933) and appropriately adapted, or by treating the whole cells with lithium acetate (Ho, H. et al., *J. Bacteriol.* 153 (1983) 1683 - 168), which favors DNA incorporation (Bianchi, M. et al., *Curr. Genet.* 12 (1987) 185 - 192). When the method involving lithium acetate is used, cell growth is performed at 28°C in 50 ml of YPD medium with agitation, and to an optical density at 600 nm (OD600) between 0.6 and 0.8. The cells are harvested by low-speed centrifugation, washed in sterile TE solution (10 mM Tris-HCl pH 7.4, 1 mM EDTA), resuspended in 3-4 ml of lithium acetate solution (0.1 M in TE) to give a cell density of about 2×10^9 c/ml and then incubated at 30°C for 1 hour with moderate agitation. 0.1 ml aliquot portions of the resulting suspension of competent cells are incubated at 30°C for 1 hour in the presence of DNA and PEG 4000 at a final concentration of 35%. After a thermal shock of 5 minutes at 42°C, the cells are washed twice with sterile water, resuspended in 0.2 ml of sterile water and transferred to 10 ml tubes. YPD medium containing 0.7% agar and kept molten at 48°C is then added (5ml) and the mixture is immediately poured onto YPD plates. After solidification, an additional overlay of 5 ml of top agar is then added. After a 18 to 24 hours incubation at 28°C, 0.16 ml of G418 solution (Geneticin 50 mg/ml, Grand Island, N.Y.) is spread onto the plates and transformants are counted after 4-5 days of additional incubation at 28°C.

Direct plating of the cells on YPD + G418 plates (i.e. omitting the use of top agar) results in the appearance of clones of larger size for the transformed K. lactic cells. However, a lower concentration of G418 (final concentration 50 µg/ml) has to be used to observe colonies, which leads to the selection of mutants partially resistant to G418 and derived from nontransformed cells. In addition, the transformation efficiency is at least ten times lower than the efficiency observed after phenotypic expression of the resistance gene for 18 to 24 hours.

E.4.2 Mitotic stability of HSA-expressing pkD1 derived plasmide under non selective growth conditions

The mitotic stability of plasmids pYG19 and pYG23 is measured at different time intervals after growth in nonselective medium; it is determined as the ratio between the final and the initial percentages of cells growing on YPD plates containing 200 µg/ml of G418. As shown in Figure 28, both plasmids are

exceptionally stable despite the high level of expression of a heterologous gene. When transformants of strain MW98-8C are grown in a non-selective medium for 40 cell generations, 40-45% of the cells have maintained these plasmids. Plasmid pYG19 shows even higher mitotic stability in *K. lactis* strain CBS633; over 80 % of the cells maintain the plasmid after 40 generations of non-selective growth.

E.4.3 pYG19 and pY623 transformants of *K. lactis* MW98-8C: HSA expression and secretion

The expression and secretion levels of *K. lactis* MW98-8C cells containing plasmids pYG19 (prepro-HSA) and pY623 (Met-HSA) are determined after different time intervals after growth at 28 °C in non-selective YPD medium and with constant agitation. The culture supernatants are obtained by two consecutive centrifugations (5 minutes at 4000 rpm in a Kontrol Hermit Z-365 K centrifuge) and then 10 minutes at 12000 rpm in order to eliminate all possible contamination by cells. A sample of the second supernatant (0.5 ml) is heated to 95 °C for 15 minutes in presence of an equal volume of sample buffer containing 0.125 M Tris-HCl, 20% glycerol, 10% 2-mercaptoethanol and 0.4 mg/ml dodecyl sulfate (SDS) and 0.4% bromophenol blue. When a concentration of the proteins present in the supernatant is desired, 0.4 ml of a 100% w/v trichloroacetic acid solution (TCA) is added to 8 ml of supernatant and the proteins are precipitated on ice for 1 hour. The precipitated material is recovered by centrifugation for 20 minutes at 15000 rpm and then resolubilized by heating to 95 °C for 15 minutes in 0.5 ml of sample buffer containing 63 mM Tris-HCl, 10% glycerol, 5% SDS and 0.2% bromophenol blue.

The intracellular expression of albumin is detected using cell extracts prepared as follows: the equivalent of 0.25 ml of a cell pellet (washed once in saline buffer) is resuspended in the same volume of lysis buffer kept on ice and containing 67 mM phosphate, pH 7.5 mM β-ME, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM leupeptin and 2 μM pepstatin A (Sigma). After addition of 0.5 ml of zirconium beads (0.5 mm diameter) stored in phosphate buffer (67 mM, pH 7) at 4 °C, the cells are ruptured by 7 consecutive 30 second periods with a cell disrupter (Biospec Mini Bead-Beater, Biospec Products) interspersed by 2 minute cooling periods on ice. In these conditions, the efficiency of cell disruption is more than 80% as judged by counting the cells under a phase-contrast microscope. The liquid fraction benefit of zirconium beads is transferred to an Eppendorf tube and the beads are washed 3 times with 0.2 ml of lysis buffer and then collected in the same Eppendorf tube. By centrifugation of the tube for 15 minutes at 4 °C and at 12000 g, a soluble protein fraction (supernatant) and an insoluble protein fraction (pellet) are thus defined. These two fractions are taken up to give the same final volume of sample buffer. These samples are then heated for 15 minutes to 95 °C and aliquots are layered on an 8.5% SDS-polyacrylamide gel preceded by a 5% stacking gel (Laemmli, U.K., Nature 227 (1970) 680 - 685), and subjected to a current of 25 mA until the bromophenol blue reaches the bottom of the gel.

Figure 29 shows the result of a typical experiment which enables an assessment to be made of the expression and secretion of albumin obtained with *K. lactis* strain MW98-8C transformed with plasmids pYG19 (prepro-HSA), pYG23 (Met-HSA) and pYG25 (vector devoid of an expression cassette), after growth in 50 ml of YPD medium without G418 at 28 °C for 68 hours. Each sample corresponds to 100 μl of original culture and enables the soluble fractions, insoluble fractions and culture supernatants to be compared after migration in 8.5% polyacrylamide gel and Coomassie Blue stain. A protein band which comigrates with commercial human albumin (Sigma) serving as a molecular weight reference, is detectable in the samples derived from the cells transformed with plasmids pYG19 or pYG23, but not with cells containing the vector pYG25 which does not contain the HSA gene. It will be noted that, whereas virtually the whole of the albumin expressed using pYG19 (prepro-HSA) is exported into the culture supernatant, all the albumin produced using plasmid pYG23 (Met-HSA) is present in the insoluble cytoplasmic protein fraction. In addition, the results in Figure 29 show that albumin excreted by the cells containing plasmid pYG19 is the only extracellular protein present in significant amounts. In the supernatant of a shake flask culture of MW98-8C transformed with plasmid pYG19 and grown in appropriate conditions (c.f. section E.4.6), the albumin concentration can reach 150 mg HSA/liter.

E.4.4 Immunological detection of the albumin produced by *K. lactis*.

The identity of the yeast proteins which co-migrate with albumin standard may be tested by immunological detection. To this end, a polyacrylamide gel is blotted onto a nitrocellulose filter (Schleicher and Schuell, 0.45 μm) using a semi-dry blotting apparatus (Biometra) in a transfer buffer containing 25 mM Tris base, 150 mM glycine and 10% methanol. The blotting time is 30 minutes using a current of

approximately 0.85 mA/cm² of gel surface. After blotting, the filter is incubated for three times 5 minutes with 50 ml of buffer A (5% skimmed milk powder, 0.2% Tween 20 in PBS buffer [137 mM NaCl, 2 mM KCl, 4.3 mM Na₂HPO₄]), followed by incubation for 30 minutes in 40 ml of buffer A containing rabbit polyclonal antibodies directed towards HSA and diluted 1:1000. After 3 rinses with buffer A, a second, biotinylated antibody recognizing the rabbit antibodies (Vestasian ABC Immuno Peroxidase Kit, Vector Laboratories), is added on the basis of one drop per 50 ml of buffer A. After incubation of the filter for 30 minutes, the filter is rinsed 3 times with buffer B (0.2% Tween 20 in PBS) and then incubated in the presence of an avidin-biotinylated peroxidase complex. The avidin-biotinylated peroxidase complex is prepared immediately before use by diluting 1 drop of each of reagents A and B of the kit in 5 ml of buffer B after incubation at 23 °C for 30 minutes. The filter is rinsed again with buffer B for 3-5 minute periods and developed for 2-3 minutes in a solution of 10 ml containing 0.02% H₂O₂ and 10 ml of a solution containing 1 mg/ml of diaminobenzidine and 0.4 mg/ml NiCl₂ in 0.1 M Tris-HCl pH 7.4. Figure 30 shows that *K. lactis* MW98-8C expresses (pYG23) and excreses (pYG19), a protein recognized by anti-HSA polyclonal antibodies. This antigenic material is specifically associated with the presence of an albumin-expressing cassette since it is not detected in cell extracts or culture supernatants from yeasts transformed with the vector without an expression cassette.

As shown in Figure 31A, the secretion of albumin takes place with relatively slow kinetics in an Ehrenmeyer. The maximum excretion levels appear to take place after 70 to 100 hours of incubation of a culture originally inoculated at 10⁵ cells/ml (Figure 31B). No significant increase or decrease is detectable between 100 and 240 hours of culture, suggesting a good stability of HSA under these growth and temperature conditions. Since there is no accumulation of proteolytically degraded material during this period, it may be concluded that no extracellular protease which might degrade the excreted albumin is present.

A graphic representation of the kinetics of excretion (A), as well as the growth curve of the strain MW98-8C transformed with plasmid pYG19 (B) is shown in Figure 32; these results show that the excretion of albumin continues after the cells have reached the stationary growth phase. This observation is in agreement with observations relating to other expression systems in yeast (Tschopp, J.F. et al. BioTechnology 5 (1987) 1305-1308).

5 10 15 20 25 30 35 40 45 50

E.4.5 Kinetics of HSA secretion

As shown in Figure 31A, the secretion of albumin takes place with relatively slow kinetics in an Ehrenmeyer. The maximum excretion levels appear to take place after 70 to 100 hours of incubation of a culture originally inoculated at 10⁵ cells/ml (Figure 31B). No significant increase or decrease is detectable between 100 and 240 hours of culture, suggesting a good stability of HSA under these growth and temperature conditions. Since there is no accumulation of proteolytically degraded material during this period, it may be concluded that no extracellular protease which might degrade the excreted albumin is present.

A graphic representation of the kinetics of excretion (A), as well as the growth curve of the strain MW98-8C transformed with plasmid pYG19 (B) is shown in Figure 32; these results show that the excretion of albumin continues after the cells have reached the stationary growth phase. This observation is in agreement with observations relating to other expression systems in yeast (Tschopp, J.F. et al. BioTechnology 5 (1987) 1305-1308).

5 10 15 20 25 30 35 40 45 50

E.4.6 Efficiency of HSA secretion with plasmids pYG44, pYG51, pYG404, and pYG58.

The efficiency of HSA secretion has been tested under control of the PGK promoter of *S. cerevisiae* (plasmids pYG19; Figures 29 to 32), a PGK/LAC4 hybrid promoter with optimized ATG context (plasmids pYG44-5 and pYG44-7; Figure 33), the PHO5 promoter of *S. cerevisiae* (plasmid pYG51; Figure 34 A), and the LAC4 promoter of *K. lactis* (plasmid pYG404; Figure 35). In addition, the secretion signal sequence (pre-region) of the killer toxin of *K. lactis* (pYG58; Figure 34 B) and the secretion efficiency of the fusion protein compared to that of the natural prepro-HSA (for details of plasmid constructions c.f. example 3). The results obtained with these constructions can be summarized as follows:

The level of HSA secreted into the culture medium when expressed from the PGK/LAC4 hybrid promoter present in plasmid pYG44 is increased two to three fold if cells are cultured in glucose-containing media to which lactose is added between mid-log and early stationary growth phase (Figure 33). This effect is not observed if the addition of lactose is replaced by an equivalent amount of glucose (data not shown). If cells are cultured in media containing lactose as sole carbon source, levels of secreted HSA are not higher as the ones obtained with glucose containing media (Figure 33 B). The lactose mediated increase of HSA secretion observed after "induction" of cells subsequent to initial cultivation in glucose, however, is not dependent on the presence of the UAS element of the LAC4 promoter of *K. lactis*. This lactose mediated increase in HSA secretion is equally found when HSA expression is directed by the PGK promoter (Figure 33 A, B) for strain MV98-8C transformants; Figure 35 for CBS2359 (transformants) or the PHO5 promoter of *S. cerevisiae* (data not shown).

5 10 15 20 25 30 35 40 45 50

55 55 55

expression is lightly regulated: no secreted HSA could be detected in culture supernatants of CBS2359 transformants when glucose was the only available carbon source (Figure 36).

Surprisingly, when cells are grown in complete medium (YPD), the efficiency of PHO5 directed HSA secretion is only slightly below the one observed with constructs containing the glycolytic PGK promoter (Figure 34 A).

The replacement of the HSA secretion signal by the pre-region of the killer toxin of K. lactis does neither influence the kinetics nor the efficiency of HSA secretion (Figure 34).

E.4.7 Efficiency of HSA secretion with integrated expression cassettes.

The PGK/prepro-HSA expression cassette present in plasmid pYG19 has been integrated into the K. lactis chromosomal DNA by the one step gene disruption method (for experimental details cf. section E3.7). As shown in figure 36, the levels of HSA secreted into the culture medium by the integrant strains (lanes 2-7) are at least twenty fold below those obtained by the pKD1 derivative pYG19 (lane 1). HSA secretion was basically the same whether integration of the expression cassette occurred at the RAD2 locus or elsewhere in the K. lactis genome (Figure 36). Furthermore, the orientation of the expression cassette with regard to the URA3 selectable marker gene did not have a significant influence on HSA secretion levels (Figure 36, lanes 2 and 3). However, a twofold increase of secreted HSA was observed with transformants which contained two integrated expression cassettes per genome (Figure 36, lane 5). In summary, the efficiency of HSA secretion appears to be directly linked to the number of copies of the structural gene carried in a transformed yeast cell.

E.4.8 Secretion of HSA in a variety of species and strains of the genus Kluyveromyces.

Plasmids pYG19, pYG221B or pKan707 have been used to transform the following strains of Kluyveromyces: ATCC16045 (K. marxianus var. bulgaricus), ATCC24178 (K. wickerhamii), ATCC12424 (K. marxianus var. marxianus), ATCC35650 (K. waltii), ATCC36906 (K. marxianus var. drosophilaeum), CBS5574 (K. marxianus var. lactis), CBS683 (K. marxianus var. lactis), and MW98-8C (K. marxianus var. lactis). Detectable levels of secreted HSA were obtained with all transformants examined, even though appreciable variations were observed among different strains. The highest levels were found using transformants of strains MW98-8C (CBS579.88) and CBS683, the lowest level was observed with strain ATCC16045 where it could only be detected using immunological methods (data not shown). Plasmids pYG19 and pYG221B mediated comparable levels of secreted HSA in all strains tested.

Example 5: PURIFICATION OF HSA SECRETED INTO THE GROWTH MEDIUM

In a typical purification experiment, strain MW98-8C transformed with plasmid pYG19 is grown in YPD medium for 72 hours under the standard conditions already described. A culture supernatant (0.5 liter) is freed from all cellular contamination by centrifugation, and incubated at 4°C for 15 minutes in the presence of 60% ethanol (V/V). The precipitate is recovered by centrifugation, redissolved in 10 ml of a solution comprising 50 mM Tris-HCl pH 8.0, and then loaded onto a Triacycl blue column (IB.F., France). The recombinant albumin is eluted from this column with 3M NaCl solution. After dialysis of the fractions containing HSA against 50 mM Tris-HCl, these fractions are purified on MONO Q column (Pharmacia), and eluted at a NaCl concentration of 0.25 M. In a final stage chromatography on Superose 12 (Pharmacia) enables the HSA to be obtained at more than 99% purity as judged from the silver staining of SDS polyacrylamide gels.

Example 6: CHARACTERIZATION OF THE SECRETED AND PURIFIED ALBUMIN

The lack of a test enabling the biological properties of albumin to be measured *in vitro* does not provide an easy mean to assess the quality of the recombinant albumin. For this reason, after purification, the albumin excreted by K. lactis is hence characterized by several physicochemical and immunological tests. These tests demonstrate that the recombinant albumin is secreted in a mature form and in its native configuration; it is not distinguishable from human serum albumin extracted from plasma with respect

to any of the criteria applied.

E.6.1 PAGE-SDS: Coomassie Blue and Silver staining.

Electrophoresis is performed on a SDS polyacrylamide gel (7.5%) (Figure 38 C) as described above, or by using the "Presto gel" system (Pharmacia, Figure 38 A). Different amounts of recombinant albumin were compared with a commercial preparation or standard albumin (Sigma) extracted from human plasma. Staining of the gel with Coomassie Blue (Figure 38 C) or with silver salts (Figure 38 A) shows the complete homogeneity of the albumin samples from yeast.

E.6.2 Isoelectric focusing.

Isoelectric focusing is performed between pH 4.0 and 7.0 (Immobiline, LKB). The isoelectric point of the recombinant HSA is identical to that of the reference human HSA ($pI \approx 4.8$).

E.6.3 Native PAGE and Immunoblotting.

Electrophoresis of the recombinant HSA in a non-denaturing polyacrylamide gel (10%), followed by blotting onto a nitrocellulose filter and immunodetection under the conditions described in section E.4.4, reveal a comigration with the standard albumin extracted from human plasma. This suggests very strongly that correct maturation of the recombinant prepro-HSA takes place during the process of secretion in K. lactis. No contamination with non-matured HSA is detectable, which implies that cleavage of the signal sequence and of the pro-sequence of HSA takes place efficiently in this yeast.

E.6.4 Molecular sieving chromatography

Molecular sieving chromatography is performed with Superose 12 (Pharmacia). The flow rate of the elution buffer (50 mM Tris-HCl pH 8.0) is maintained at 1 ml/minute. The concentrations of recombinant albumin and of reference albumin are 0.4 and 1 mg/ml respectively. The elution of albumin is detected by measuring the absorbance at 280 nm. In both cases, the elution volume is identical (11.5 ml).

E.6.5 Anion exchange chromatography

The recombinant albumin is eluted from a Mono O HR 5/5 column (Pharmacia) at a concentration of 0.31 M NaCl, as is the reference albumin derived from human plasma. Figure 39 shows the chromatographic profiles obtained for injections of 100 μ l of recombinant HSA (0.4 mg/ml) and of reference HSA (0.5 mg/ml) using a column equilibrated with 50 mM Tris-HCl pH 8.0, and at a constant flow rate of 1 ml/minute.

E.6.6 Reversed-phase chromatography

The behavior of the yeast albumin in reversed-phase is analyzed on a Nucleosil (C4) column with buffers A (water containing 0.1% trifluoroacetic acid (TFA)) and B (acetonitrile containing 0.1% TFA). Figure 40 shows the elution, with a gradient of 20 to 80% of B in A, of the yeast albumin, which exhibits the same retention time as the reference albumin.

E.6.7 Amino acid composition

The amino acid composition of the recombinant HSA is determined by reversed-phase chromatography after acid hydrolysis (6 N HCl) and phenylisothiocyanate derivatization. The results obtained by this method show clearly an identical composition to that of the reference albumin derived from human plasma.

E.6.8 N-Terminal sequence

The use of an automated apparatus for Edman degradation (Applied Biosystems) shows that the N-terminal sequence of the HSA secreted by *K. lactis* is Asp-Ala-His-Lys-Ser-Glu-Vai-Ala-His-Arg-Phe-Lys-Asp-Lau-Gly. The determination of this sequence hence confirms the results derived from the native polyacrylamide gel electrophoresis experiments, and demonstrates the correct and complete maturation of the albumin secreted by *K. lactis*.

E.6.9 Tryptophan fluorescence

After excitation at 295 nm, the fluorescence emission of the single tryptophan shows the same profile for both recombinant and plasma albumins (maximum at 337 nm), indicating an identical hydrophobic environment around this amino acid (Figure 41).

E.6.10 Thermal stability

The reference albumin (Sigma) and the albumin secreted by the yeast have identical stability kinetics at 75°C (Figure 42), which suggests that the bonds stabilizing the protein structure (hydrophobic interactions and disulfide bonds) are identical in both cases.

E.6.11 Reactivity towards monoclonal antibodies

The antigenicity of the recombinant albumin was studied with different monoclonal antibodies directed towards human albumin. Figure 43 shows their specificity: the antibodies HA10, HA11 and HA13 correspond to epitopes whose conservation requires the integrity of the whole molecule. The antibodies HA21, HA6, HA4, HA3, HA8 and HA1 correspond to epitopes localized along the peptide chain from the N-terminal to the C-terminal end (Doyen, N. et al., Mol. Immun. 22 (1985) 1 - 10; Lapresle, C., Anal. Biochem. 174 (1988) 308-312).

The test employed is an ELISA (Enzyme-Linked Immuno-Sorbent Assay) inhibition test. The different antibodies are adsorbed on polystyrene plates and a binding curve of albumin labeled with alkaline phosphatase is established for each antibody. The saturation curves for each antibody have a sigmoid appearance, and the amount of labeled albumin corresponding to 50% binding was chosen to study the inhibition by each antibody.

Inhibition was carried out with the sample of recombinant albumin and compared with that obtained with a sample of plasma albumin (Sigma). The panels A, B, and C of Figure 44 shows that, for the nine antibodies tested, the recombinant albumin is as inhibitory as native albumin. Given the extreme sensitivity of the test, the differences observed are not likely to be significant.

The structural gene encoding the recombinant IL-1 β is thus available as a EcoRI-HindIII restriction fragment contained in a derivative of plasmid pUC19 designated pC131 (Figure 46).

E.7.2 Construction of a secretion vector containing IL-1 β under the control of the PGK promoter of *S. cerevisiae* and the killer toxin secretion signal of *K. lactis*

A DNA fragment corresponding to the secretion signal ("pre" region) of the toxin gene of killer plasmid k1 of *K. lactis* (Stark, M.J.R. and Boyd, A., EMBO J. 5 (1986) 1955-2002) was assembled from the following two synthetic oligodeoxynucleotides:

SQa : 5'-AATTCTTACATTATTTACATTATTTGCTTCAGGTAAAG-3'
SQb : 5'-AATTCTTACCTTGACAGAACAAAATATGTAATAATATCAT-3'

These two oligodeoxynucleotides were hybridized, thereby reconstituting the signal sequence of the killer toxin followed by a dipeptide LysArg which represents a potential cleavage site recognizable by an endopeptidase produced by the KEX1 gene of *K. lactis* (Tanguy-Rougeau, C. et al., FEBS letters 234 (1988) 464-470). This sequence is flanked by cohesive termini compatible with those of an EcoRI site. In this construction, only the terminus situated downstream from the signal sequence can regenerate a functional EcoRI site. This DNA fragment was cloned in the desired orientation into the single EcoRI site of plasmid pEPGK1, thereby generating plasmid pSPGK1 (Figure 45). pEPGK1 contains a Sall-BamHI cassette containing the promoter and terminator of the PGK gene of *S. cerevisiae* identical to that present in plasmid p'G12 (already described in Example 3) with the exception of the HindIII cloning site which has been replaced by an EcoRI site (see the sequence given in Figure 45). As a result of the strategy used, plasmid pSPGK1 possesses a unique EcoRI site permitting the cloning of structural genes downstream from the synthetic signals sequence.

In order to obtain the structural gene encoding IL-1 β in the form of an EcoRI restriction fragment, plasmid pC131 (see section E.7.1) was linearized with the enzyme HindIII, treated with the Klenow fragment of *E. coli* DNA polymerase I to render the termini blunt-ended and ligated with a synthetic EcoRI linker (5'-GAATTCC-3'). After cleavage with the enzyme EcoRI, the fragment corresponding to Met-IL-1 β was purified by electropelusion and cloned into the EcoRI site of plasmid pSPGK1 to generate plasmid pSPGK-IL17 (Figure 46). In this construction, the IL-1 β coding sequence is in translational frame with the secretion signal derived from the killer toxin. The beginning of the mature IL-1 β gene (AlaPovA-Val) is separated from the cleavage site recognized by the signal peptidase (GlnGly \wedge) by the propeptide LysArgLysMet.

Plasmid pSPGK-IL17 is the source of a Sall-HindIII restriction fragment corresponding to the expression cassette (PGK promoter/secretion signal-IL-1 β /PGK terminator); this fragment was purified by electroelution and cloned into plasmid pCXJ1, cleaved by the same enzymes to generate plasmid pSPGK-IL21 (Figure 47). Plasmid pUCAK (Pharmacia) is the source of the gene for resistance to gentamicin (G418), which was purified by electropelusion as a Sall restriction fragment. This fragment was then cloned into the Sall site of plasmid pSPGK-IL21 to generate the IL-1 β expression plasmid pSPGK-IL31 (Figure 48).

E.7.3 Construction of a secretion vector containing IL-1 β under the control of the PHO5 promoter of *S. cerevisiae*

The importance of the PHO5 promoter lies in the fact that it represents an inducible expression system in *K. lactis*; as in *S. cerevisiae*, this promoter is repressed at high inorganic phosphate concentration and induced in medium lacking phosphate (Chen, X.J. and Fukuhara, H., Gene 369 (1998) 181-192).

the EcoRI fragment corresponding to the secretion signal of the toxin gene of *K. lactis* killer plasmid k1 (see section E.7.2) was cloned in the desired orientation into the EcoRI site of plasmid pEPHO (see section E.3.4) to generate plasmid pSPHO4 (Figure 49). The EcoRI restriction fragment corresponding to Met-IL-1 β was purified from plasmid pSPGK-IL17 and cloned into the EcoRI site of plasmid pSPHO4 to generate plasmid pSPHO-IL14 (Figure 50). This plasmid is the source of a Sall-HindIII restriction fragment corresponding to the expression cassette (PHO5 promoter/secretion signal-IL-1 β /PHO5 terminator); this fragment was purified by electropelusion and cloned into plasmid pCXJ1 cut by the same enzymes to generate plasmid pSPHO-IL23 (Figure 51). The Sall restriction fragment containing the gene for resistance to gentamicin (G418) was purified by electropelusion from plasmid pUC4K and then cloned into the Sall site of plasmid pSPHO-IL23 to generate the IL-1 β expression plasmid pSPHO-IL35 (Figure 52).

Example 7: YEAST EXPRESSION VECTORS CONTAINING A CASSETTE FOR THE SECRETION OF INTERLEUKIN-1 β .**E.7.1 Synthesis of the IL-1 β structural gene**

The chemical synthesis of the structural gene corresponding to IL-1 β has already been described (Jung, G. et al., Ann. Inst. Pasteur/Microbiol. 139 (1988) 129-148); in brief, the structural gene was assembled from 14 synthetic oligodeoxynucleotides as a NdeI-HindIII restriction fragment. Compared with the sequence published by Auron et al. (Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 7907-7911), the synthetic gene used in the present example contains the following modifications: i) change of the AGC codon (Ser) at position 483 for the TCC codon (Tyr); ii) substitution of a T by a C at position 508 to eliminate the NdeI site; iii) creation of an EcoRI site at position 435 (GAATTCATATG), including the translation initiation methionine (bold letters); iv) addition of the sequence AAGCTT at position 896 in order to introduce a HindIII site immediately downstream from the translation termination codon.

E.7.4 Mitotic stability of IL-1 β -expressing pKD1 derived plasmids under non-selective growth conditions

The mitotic stability of plasmids pSPGK-IL31 and pSPHO-IL35 was defined as the percentage of cells which have retained the capacity to grow on minimum selective medium (not supplemented with uracil) after growth of a defined number of generations in non-selective medium (YPD). As shown in Figure 53. These 2 plasmids are remarkably stable, although there is a different stability between the construction using the PGK promoter compared with the construction containing the PHO5 promoter: after 40 cell generations in a non-selective medium, 75% (promoter induced) to 93% (promoter not induced) of the cells have maintained plasmid pSPHO-IL35, whereas plasmid pSPGK-IL31 is retained in only 44% of the cells. These results show the importance of an inducible expression system uncoupling the growth phase of a yeast culture from the phase of production of a potentially toxic heterologous protein, from the standpoint of the industrial exploitation of expression vectors derived from plasmid pKD1.

E.7.5 Secretion of IL-1 β .

The constructions pSPGK-IL31 and pSPHO-IL35 were transferred into *K. lactis* strain MW98-8C according to the techniques already described. After selection of the transformants in minimum medium devoid of uracil, a few clones were inoculated in YPD medium containing 200 μ g/ml of G418 and their capacity to secrete IL-1 β was studied. Samples of the culture supernatant were treated with ethanol (60 minutes at -20°C at a final concentration of 60%) in order to precipitate the secreted IL-1 β . The precipitated material was recovered by centrifugation (20 minutes at 15000 g) and resuspended in sample buffer by heating to 95°C for 15 minutes in one tenth of the original volume before being deposited on a 12.5% polyacrylamide gel (Laemmli, U.K., Nature 227 (1970) 680 - 685). After electrophoresis of the samples, IL-1 β secretion was detected either in denaturing gels stained with Coomassie Blue (Figure 54) or by immunological detection after blotting the gel onto nitrocellulose membranes and using polyclonal antibodies directed towards human IL-1 β (Genzyme), with or without prior treatment of the samples with endoglycosidase H according to the directions of the manufacturer (Boehringer Mannheim).

The immunological signal obtained from the supernatants of the yeasts transformed with the construction corresponding to IL-1 β is sensitive to treatment with endoglycosidase H (decrease in the apparent molecular weight from 24 kD to 19 kD), demonstrating that the recombinant IL-1 β is glycosidated. This result is in agreement with the presence of a potential N-glycosylation site in the N-terminal portion of IL-1 β (Asn at position 7).

2 ml of culture supernatant of *K. lactis* MW98-8C, transformed with plasmid pSPHO-IL35 and grown in an Erdmanner medium lacking inorganic phosphate (Chen, X.J. and Fukuhara, H., Gene 369 (1998) 181-192, were directly applied to a reversed-phase column in order to purify the secreted IL-1 β ; the elution peak corresponding to 35% of acetonitrile was collected and corresponds to the recombinant interleukin. The quantity of material purified by this technique was determined by spectrophotometry and allows to estimate that IL-1 β is secreted by *K. lactis* at the level of 35 mg per liter of culture supernatant.

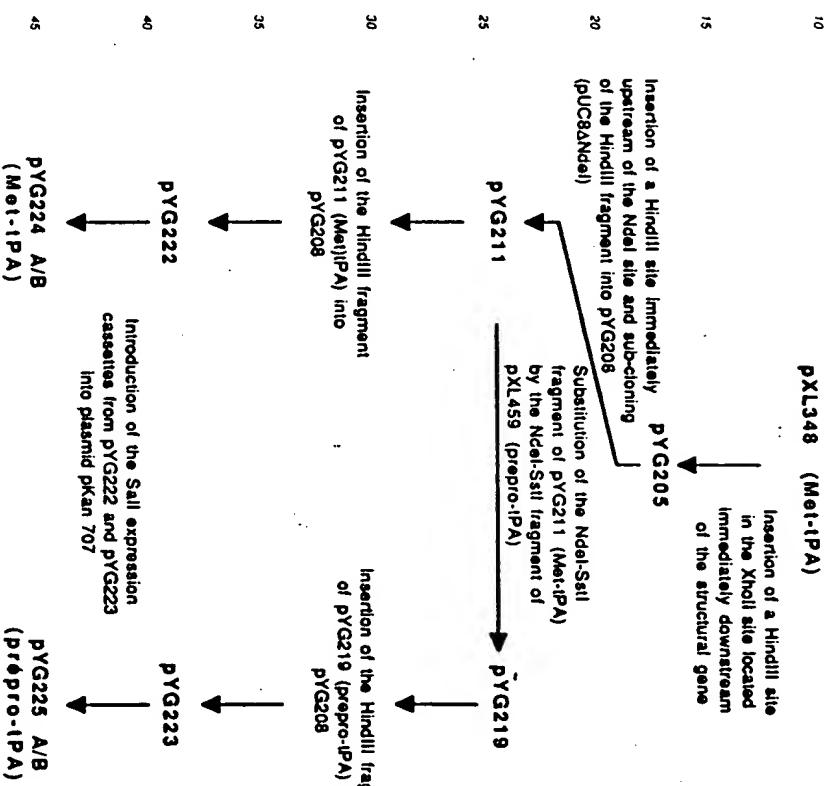
Sequencing of the N-terminal portion of the purified IL-1 β (automated Edman degradation, Applied Biosystems) indicates the total absence of the "pre" region of the killer toxin and hence the effective maturation by *K. lactis* strain MW98-8C at the Gingly⁺ cleavage site which would be recognized by a yeast signal peptidase (Stark, M.J.R. and Boyd, A., EMBO J. 5 (1986) 1955-2002). As a result of the strategy used in the construction of the IL-1 β -expressing cassette present in plasmid pSPHO-IL35, the N-terminal end of the recombinant IL-1 β cannot be identical to that of mature human IL-1 β : the arrangement of the first 11 residues determined by microsequencing (LysArgLeuIleAlaProValArgSerLeu) confirms the presence of several additional amino acids (LysArgLeuIleMet) upstream from the sequence corresponding to mature human IL-1 β , and demonstrates, in particular, that the potential site of cleavage by the enzyme KEX1 of *K. lactis* (LysArg⁺) is not recognized in this sequence context. The creation by site directed mutagenesis of a correct junction between the secretion signal for the killer toxin and the structural gene for mature IL-1 β (Gingly⁺ Alaproval) should hence enable a correctly matured secreted IL-1 β to be obtained.

Example 8: YEAST EXPRESSION VECTORS CONTAINING A CASSETTE FOR THE EXPRESSION AND SECRETION OF IPA

E.8.1 Construction of vectors for Met-IPA expression and prepro-IPA secretion under control of the PGK promoter of *S. cerevisiae*

The structural gene encoding IPA was isolated from plasmids pX1348 (Met-IPA) and pX1459 (prepro-IPA) (Sarmientos, P. et al., Biotechnology 7 (1989) 495-501), the restriction maps of which are shown in Figure 55.

Starting from plasmid pX1348 (Met-IPA), plasmids pYG224 A/B (Met-IPA) and pYG225 A/B (prepro-IPA) are derived according to the following diagram:



Plasmid pX1348 is digested by the enzyme *Xba*I, and the restriction mixture is ligated with a phosphorylated *Xba*I-HindIII adaptor (synthetic oligodeoxynucleotide 5'-GATCAAGCTT-3'); the mixture is then digested by the enzyme *Hind*III, and the restriction fragment encoding Met-IPA is cloned into the *Hind*III site of the vector pUC8 to generate the intermediate plasmid pYG205. This plasmid is cut with the enzyme *Nde*I and ligated with an equimolar mixture of the phosphorylated synthetic oligodeoxynucleotides 5'-TAACGTTCA-3' and 5'-ATGAGCT-3' which constitute a *Nde*I-HindIII adaptor; after *in vitro* ligation, the mixture is digested by *Hind*III and the restriction fragment encoding Met-IPA is purified by electroselution and cloned into the *Hind*III site of the vector pYG208 to generate plasmid pYG211. The vector pYG208 corresponds to plasmid pUGC in which the unique *Nde*I site has been destroyed by cleavage with the enzyme *Nde*I and filling-in of the cohesive termini with the Klenow fragment of *E. coli* polymerase I before

self-ligation of the vector, plasmid PYG211 hence contains a HindIII fragment encoding Met-tPA in which there is a single MdeI restriction site localized at the ATG initiation codon of Met-tPA. In plasmid PYG211, the nucleotide sequence upstream from the translation initiation codon (bold letters) is as follows: 5'-AAGCTTCATATG...3'.

Plasmid pYG211 is the source of plasmid PYG219, which contains a HindIII fragment encoding preproto-tPA. This plasmid was constructed by substituting the NdeI-SstI fragment of PYG211 by the NdeI-SstI fragment of plasmid pX459, which contains the N-terminal portion of preproto-tPA (Figure 55); plasmids pYG211 (Met-tPA) and PYG219 (preproto-tPA) are hence strictly isogenic, except that plasmid PYG211 encodes the mature form of tPA immediately preceded by the initiation codon, whereas, in plasmid pYG219, the mature tPA sequence is preceded by its preproto natural exportation sequence.

The HindIII fragments encoding Met-tPA and preproto-tPA were purified by electroelution from plasmids pYG211 and PYG219, and cloned in the correct orientation into the vector pYG208 (c.f. Figure 19A) cut by the same restriction enzyme, which generates the intermediate plasmids PYG222 and PYG223. These plasmids contain an expression cassette available as a Sall restriction fragment which was then cloned into the vector pKan707 to generate plasmids pYG224 A/B (Met-tPA) and PYG225 A/B (preproto-tPA). The A and B orientations of the Sall cassettes with respect to the vector were defined as above for plasmids PYG221 A/B (preproto-HSA) (c.f. section E.3.2).

E.8.2 Secretion of tPA

The construction PYG224 A/B (Met-tPA) and PYG225 A/B (preproto-tPA) were transferred, in particular, into K. lactic strain MW98-8C according to the techniques described in section E.4.1. After selection of the transformed cells in the presence of G418, a few clones were inoculated in selective YPD medium, and their capacity for expressing and secreting tPA was studied according to the following techniques: (i) in Coomassie blue stained SDS polyacrylamide gels; (ii) by immunological detection after blotting of the gel onto nitrocellulose membrane and use of primary antibodies directed towards human tPA (Biopool), with or without prior treatment of the cell fractions with deglycosylating enzymes, endoglycosidase H or glycoproteinase F, which were used according to the directions of the manufacturer (Boehringer Mannheim); (iii) in a fibrin plate assay on indicator plates of the following composition: 1% agarose, 0.1% fibrinogen (KabiVitrum), 0.2 unit/ml thrombin (Sigma), dissolved in PBS buffer.

The results of these experiments demonstrate that no specific immunological signal is detectable in the untreated culture supernatants (construction PYG225B), or in the cell extracts. The detection method cannot be called into question, since the constructions pYG224 A/B (Met-tPA) give a strong specific signal (between 1 and 10 mg/liter of culture) in the cell extracts and more especially in the fraction corresponding to the insoluble proteins.

The use of deglycosylating enzymes appears as a prerequisite for the immunological detection of the preproto-tPA expressed and secreted by K. lactic under the control of the PGK promoter of S. cerevisiae. This observation may be explained by an heterogeneous hyperglycosylation of this protein when expressed in this strain under control of the PGK promoter. In particular, this hyperglycosylation could be responsible for the weak antigenicity of the yeast recombinant tPA expressed from the PGK promoter.

Figure 58 demonstrates the presence of an enzyme activity in the culture supernatants of K. lactic MW98-8C transformed with plasmid pYG225B (preproto-tPA), whereas it is absent from the culture supernatants of strain MW98-8C transformed with plasmids pKan707 (control vector) or pYG224 A/B (Met-tPA). The detection of a secreted tPA activity hence demonstrates that the preproto-sequence of tPA is functional in K. lactic MW98-8C. The test of tPA activity on indicator plates also demonstrates the presence of activity in the intracellular fractions of strain MW98-8C transformed with the construction Met-tPA (result not documented).

E.8.3 Influence of the host strain on tPA secretion

The choice of a host cell is a fundamental factor as regards the levels of tPA secretion by yeasts capable of replicating derivatives of plasmid pKan707. The capacity of yeasts of the genus Kluyveromyces to secrete tPA cloned into the vector pKan707 was tested as above on fibrinogen-agar indicator plates. K. lactis strains ATCC 34609 and ATCC 34610, ATCC 38906 (K. drosophilae), and K. fragilis ATCC 38534 and ATCC 36907 have a potential for tPA secretion of the same order of magnitude as that of K. lactic strain MW98-8C, whereas tests carried out under the same conditions on strains ATCC 12424 (K. fragilis)

ATCC 24178 (K. wickerhamii) and ATCC 56500 (K. wallii) did not enable tPA activity to be detected in the culture supernatants.

5 E.9: YEAST EXPRESSION VECTORS CONTAINING A CASSETTE FOR THE EXPRESSION AND SECRETION OF TIMP

E.9.1 Construction of vectors for expression and secretion of TIMP under control of the PGK promoter of S. cerevisiae

The complete structural gene encoding TIMP was isolated from plasmid pPV2-TIMP. This vector consists of the TIMP cDNA available as a HindIII restriction fragment (Kaczmarek, M. et al., Bio/Technol. (1987) 5:95-598). This restriction fragment was purified by electroporation and cloned in the correct orientation into the HindIII site of the construction intermediate pYG208 (c.f. Figure 19A) to generate plasmid pYG220. This plasmid is the source of a Sall fragment corresponding to the expression cassette (P G K promoter/TIMP/PGK terminator) which was cloned into the Sall site of the vector pKan707 (c.f. Figure 19) to generate the expression plasmids pYG226 A/B, which differ from one another only in the orientation of this Sall fragment with respect to the vector. The A and B orientations of plasmids pYG226 A/B were defined as above for plasmids pYG221 A/B (preproto-HSA), pYG224 A/B (Met-tPA) and pYG225 A/B (preproto-tPA).

E.9.2 Secretion of TIMP

The construction pYG226 A/B (TIMP) were transferred into K. lactic strain MW98-8C according to the techniques already described. After selection of the transformed cells in the presence of G418, a few clones were inoculated in selective YPD medium (G418, 200 µg/ml) and their capacity to express and secrete TIMP was studied, either in Coomassie blue stained SDS polyacrylamide gels, or by immunological detection after blotting of the gel onto the nitrocellulose membranes and use of polyclonal antibodies directed towards human TIMP (Rhône-Poulenc Santé, with or without prior treatment of cell fractions with endoglycosidase H.

An immunological signal corresponding to TIMP is detectable in the non-deglycosylated intracellular extracts (insoluble protein supernatants), but not in the non-deglycosylated culture supernatants (Figure 57). This immunological signal is sensitive to treatment with deglycosylating enzymes, demonstrating that the yeast recombinant TIMP is N-glycosylated. The secretion of recombinant TIMP is demonstrated after in vitro deglycosylation of a culture supernatant of strain MW98-8C transformed with plasmid pYG226B (Figure 57), indicating that at the natural exportation sequence of TIMP is functional in K. lactic MW98-8C.

Note

A sample of strain MW98-8C has been deposited at the Centraalbureau voor Schimmelcultuuren (CBS) at Baarn in the Netherlands according to the provisions of the Treaty of Budapest, where it was registered on 16.05.1988 under number CBS 579.88.

Claims

50 1. A method for the microbiological preparation of human serum albumin (HSA) or one of its variants, wherein a yeast capable of providing for the stable maintenance of an expression cassette containing at least one selectable marker for the transformed yeasts, the DNA of the structural gene for HSA or for said variant under the control of sequences permitting its expression in yeast and, where appropriate, excretion of the protein encoded by this gene into the growth medium, is grown.
55 2. The method as claimed in claim 1, wherein the expression cassette is integrated in the yeast genome.
56 3. The process as claimed in claim 1, wherein the expression cassette forms part of a plasmid containing a replication system functioning in yeast and providing for the stable maintenance of said

cassette in this yeast.

4. The method as claimed in one of claims 1, 2 or 3, wherein the yeasts are chosen from the genera

Saccharomyces and Kluyveromyces.

5. The method as claimed in one of claims 1, 2 or 3, wherein the yeast is chosen from all varieties of

Kluyveromyces marxianus.

6. The method as claimed in one of claims 1, 2 or 3, wherein the yeast is chosen from all varieties of

Kluyveromyces marxianus.

7. The method as claimed in one of claims 1, 2 or 3, wherein the yeast is Kluyveromyces marxianus var. lactis.

8. The method as claimed in one of claims 3 to 7, wherein the replication system functioning in yeast is all or part of plasmid pKD1 originally isolated from Kluyveromyces marxianus var. drosophilaeum, or all or part of the 2 μ plasmid isolated from Saccharomyces cerevisiae, or a combination of elements derived from plasmid pKD1 and from the 2 μ plasmid.

9. The method as claimed in one of claims 1, 2 or 3, wherein the replication system functioning in yeast is all or part of the pKD1 sequence.

10. A method for the preparation of a specified protein, in which method a yeast of the genus Kluyveromyces transformed with an expression plasmid comprising:

genes A, B and C of plasmid pKD1,

the inverted repeats of plasmid pKD1,

the stability locus of plasmid pKD1,

the origin of replication of pKD1 and an expression cassette containing a DNA encoding the structural gene for said protein under the control of sequences permitting its expression in said yeast,

a selectable marker for the transformed yeast,

and, optionally, an origin of replication and a selectable marker for Escherichia coli.

11. The method as claimed in one of claims 8 to 10, wherein the expression cassette and the selectable marker are inserted in the EcoRI site of pKD1.

12. The method as claimed in one of claims 8 to 11, wherein the expression cassette and the selectable marker are inserted in a region of 197 nucleotides defined by the SacI and MstII sites of pKD1, or the SphI site of pKD1.

13. The method as claimed in one of claims 1 to 12, wherein the sequences permitting the expression of the structural genes are chosen from the promoters derived from genes of yeasts of the genus Saccharomyces or Kluyveromyces.

14. The method as claimed in claim 13, wherein these promoters are derived from the glycolytic genes of yeasts of the genus Saccharomyces or Kluyveromyces.

15. The method as claimed in claim 13, wherein the sequences permitting the expression of the structural genes are chosen from the genes encoding phosphoglycerate kinase (PGK), glyceraldehyde-3-phosphate dehydrogenase (GPD), endonases (ENO), alcohol dehydrogenases (ADH), lactase (LAC α) or acid phosphatase (PHO5).

16. The method as claimed in claim 13, wherein the sequences permitting the expression of the structural genes are chosen from the genes encoding phosphoglycerate kinase (PGK) or acid phosphatase (PHO5).

17. The method as claimed in one of claims 1 to 16, wherein the sequences encoding said protein are preceded by a protein-exportation sequence which is chosen from the natural N-terminal leader of the said protein.

18. The method as claimed in one of claims 1 to 17, wherein the sequence encoding said protein is preceded by an exportation sequence different from the natural sequence.

19. The method as claimed in claim 18, wherein the sequence encoding the exportation signal of said protein is the sequence obtained from yeast genes encoding the alpha pheromone or killer toxin.

20. The method as claimed in one of claims 1 to 19, wherein the protein is chosen from:

- interleukins
- albumins
- tPA
- TIMP.

21. The method as claimed in claim 20 wherein the sequence encoding the specified protein is chosen from the sequences encoding:

- IL-1 β

- prepro-HSA
- Met-HSA
- prepro-PA
- Met-PA and

TIMP.

22. The method as claimed in one of claims 1 to 19, wherein the sequence permitting the excretion of HSA is the prepro natural terminal leader of albumin.

23. The method as claimed in one of claims 1 to 22, wherein the selectable markers for the transformed yeasts are chosen from the genes providing resistance to antibiotics or to copper ions.

24. The method as claimed in claim 23, wherein the selectable sequence is a gene providing resistance to G418.

25. The method as claimed in claim 24, wherein the selectable sequence is a fusion between the ORF1 promoter of the linear plasmid k1 of K. lactis and the aminoglycoside phosphotransferase of the transposon Tn903.

26. The method as claimed in one of claims 1 to 25, wherein the selectable marker is a gene complementing auxotrophies.

27. The method as claimed in claim 1, wherein the strain transformed with a plasmid which contains plasmid pKD1, prepro-HSA DNA, the transcription initiation and termination sequences of the gene encoding phosphoglycerate kinase and the sequences of a fusion between the ORF1 promoter of the linear plasmid k1 of K. marxianus var. lactis and the 3'-aminoglycoside phosphotransferase gene of Tn903.

28. The method as claimed in claim 1, wherein the strain is transformed with a plasmid which contains plasmid pKD1, Met-HSA DNA, transcription initiation and termination sequences of the gene encoding phosphoglycerate kinase and the sequences of a fusion between the ORF1 promoter of the linear plasmid pKD1, Met-HSA DNA, transcription initiation and termination sequences of the gene encoding phosphoglycerate kinase and the 3'-aminoglycoside phosphotransferase gene of Tn903.

29. The method as claimed in one of claims 1 to 28, wherein the strain is of the species K. marxianus, K. wickerhamii, K. wallii.

30. The method as claimed in claim 29, wherein the strain is of the species K. bulgaricus, K. marxianus var. marxianus var. lactis and the 3'-aminoglycoside phosphotransferase gene of Tn903.

31. The method according to anyone of claims 1 to 30, wherein yeasts are cultured in glucose containing medium to which lactose is added between mid-log and early stationary growth phase.

32. Human serum albumin, when obtained by the method as claimed in one of claims 1 to 31.

33. The application of human serum albumin as claimed in claim 32, by way of a medicinal product.

34. The method as claimed in claim 33, wherein the medicinal product is a pharmaceutical composition.

35. The method as claimed in claim 33, wherein the pharmaceutical composition is a parenteral formulation.

36. The method as claimed in claim 33, wherein the pharmaceutical composition is a topical formulation.

37. The method as claimed in claim 33, wherein the pharmaceutical composition is a nasal formulation.

38. The method as claimed in claim 33, wherein the pharmaceutical composition is a pulmonary formulation.

39. The method as claimed in claim 33, wherein the pharmaceutical composition is a rectal formulation.

40. The method as claimed in claim 33, wherein the pharmaceutical composition is a vaginal formulation.

41. The method as claimed in claim 33, wherein the pharmaceutical composition is a buccal formulation.

42. The method as claimed in claim 33, wherein the pharmaceutical composition is a parenteral formulation.

43. The method as claimed in claim 33, wherein the pharmaceutical composition is a topical formulation.

44. The method as claimed in claim 33, wherein the pharmaceutical composition is a nasal formulation.

45. The method as claimed in claim 33, wherein the pharmaceutical composition is a pulmonary formulation.

46. The method as claimed in claim 33, wherein the pharmaceutical composition is a rectal formulation.

47. The method as claimed in claim 33, wherein the pharmaceutical composition is a vaginal formulation.

48. The method as claimed in claim 33, wherein the pharmaceutical composition is a buccal formulation.

49. The method as claimed in claim 33, wherein the pharmaceutical composition is a parenteral formulation.

50. The method as claimed in claim 33, wherein the pharmaceutical composition is a topical formulation.

51. The method as claimed in claim 33, wherein the pharmaceutical composition is a nasal formulation.

52. The method as claimed in claim 33, wherein the pharmaceutical composition is a pulmonary formulation.

53. The method as claimed in claim 33, wherein the pharmaceutical composition is a rectal formulation.

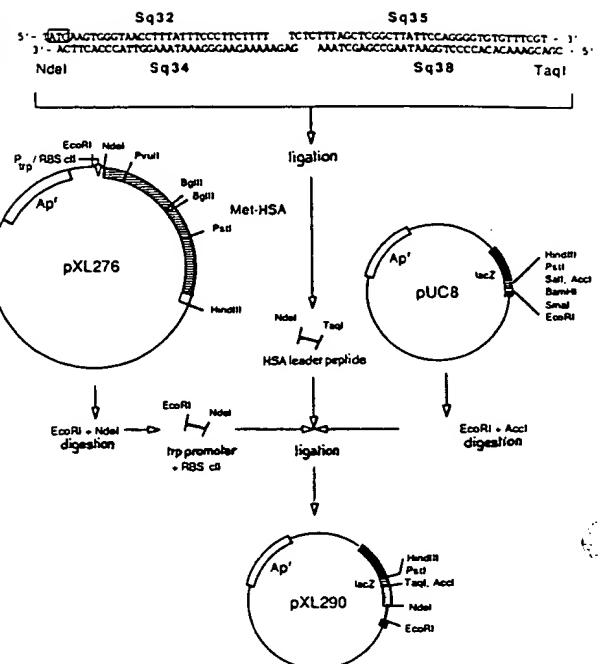


FIG. 2

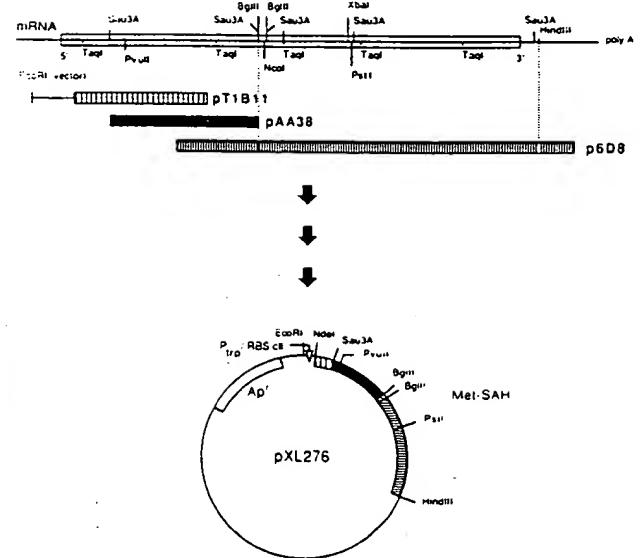
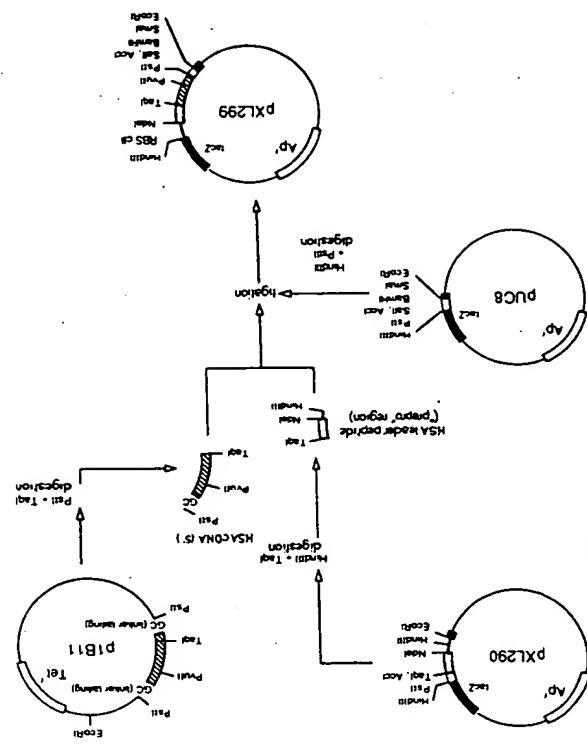


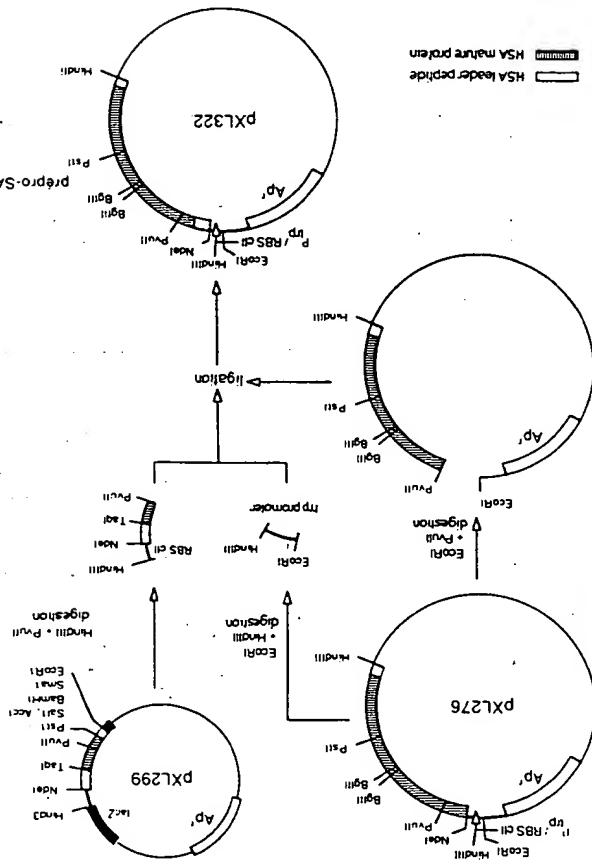
FIG. 1

FIG. 3



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FIG. 4



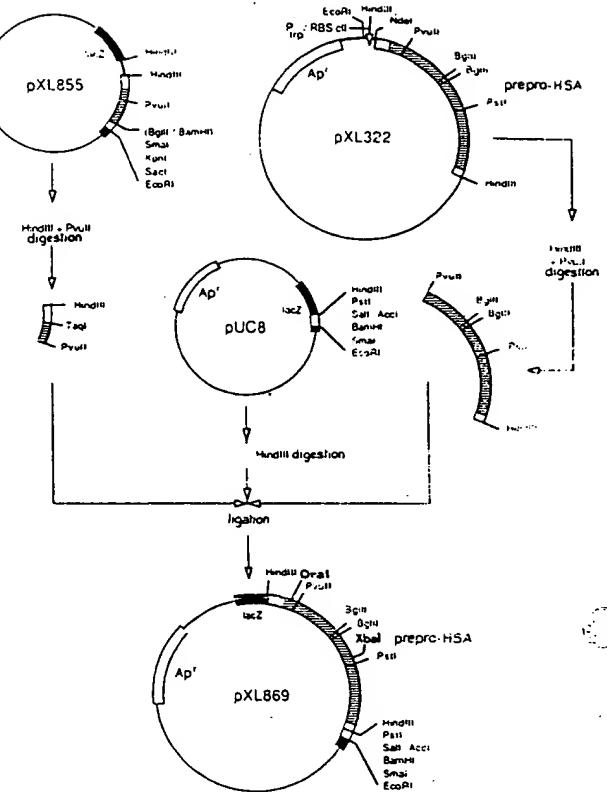


FIG.6

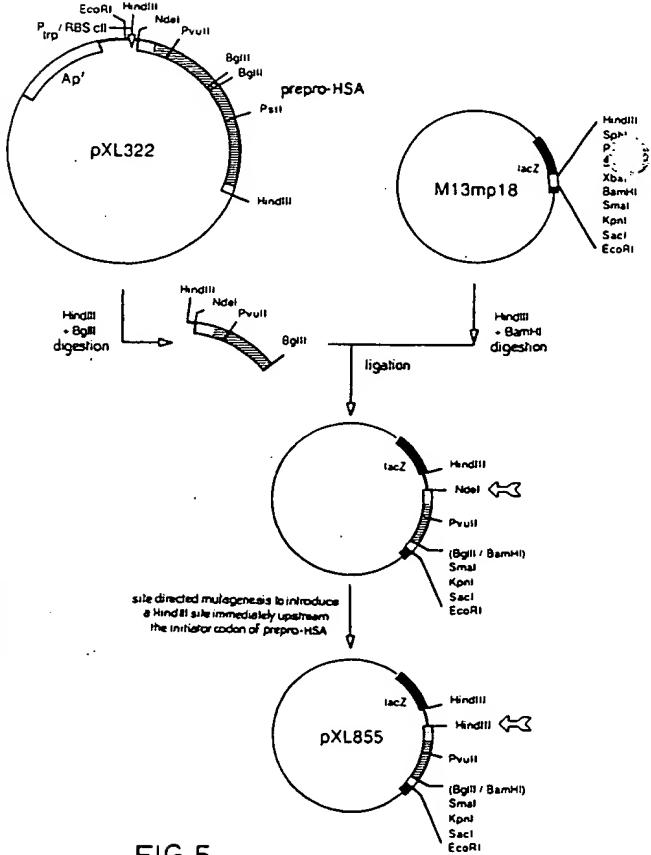


FIG.5

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9.	1286	1292	1300	1312	1325	1336	1348
10.	1354	1365	1376	1388	1399	1410	1421
11.	1421	1432	1443	1454	1465	1476	1487
12.	1521	1532	1543	1554	1565	1576	1587
13.	1611	1612	1613	1614	1615	1616	1617
14.	1691	1692	1693	1694	1695	1696	1697
15.	1781	1782	1783	1784	1785	1786	1787
16.	1871	1872	1873	1874	1875	1876	1877
17.	1961	1962	1963	1964	1965	1966	1967
18.	2051	2052	2053	2054	2055	2056	2057
19.	2141	2142	2143	2144	2145	2146	2147
20.	2231	2232	2233	2234	2235	2236	2237
21.	2321	2322	2323	2324	2325	2326	2327
22.	2411	2412	2413	2414	2415	2416	2417
23.	2501	2502	2503	2504	2505	2506	2507
24.	2591	2592	2593	2594	2595	2596	2597
25.	2681	2682	2683	2684	2685	2686	2687
26.	2771	2772	2773	2774	2775	2776	2777
27.	2861	2862	2863	2864	2865	2866	2867
28.	2951	2952	2953	2954	2955	2956	2957
29.	3041	3042	3043	3044	3045	3046	3047
30.	3131	3132	3133	3134	3135	3136	3137
31.	3221	3222	3223	3224	3225	3226	3227
32.	3311	3312	3313	3314	3315	3316	3317
33.	3401	3402	3403	3404	3405	3406	3407
34.	3491	3492	3493	3494	3495	3496	3497
35.	3581	3582	3583	3584	3585	3586	3587
36.	3671	3672	3673	3674	3675	3676	3677
37.	3761	3762	3763	3764	3765	3766	3767
38.	3851	3852	3853	3854	3855	3856	3857
39.	3941	3942	3943	3944	3945	3946	3947
40.	4031	4032	4033	4034	4035	4036	4037
41.	4121	4122	4123	4124	4125	4126	4127
42.	4211	4212	4213	4214	4215	4216	4217
43.	4301	4302	4303	4304	4305	4306	4307
44.	4391	4392	4393	4394	4395	4396	4397
45.	4481	4482	4483	4484	4485	4486	4487
46.	4571	4572	4573	4574	4575	4576	4577
47.	4661	4662	4663	4664	4665	4666	4667
48.	4751	4752	4753	4754	4755	4756	4757
49.	4841	4842	4843	4844	4845	4846	4847
50.	4931	4932	4933	4934	4935	4936	4937
51.	5021	5022	5023	5024	5025	5026	5027
52.	5111	5112	5113	5114	5115	5116	5117
53.	5201	5202	5203	5204	5205	5206	5207
54.	5291	5292	5293	5294	5295	5296	5297
55.	5381	5382	5383	5384	5385	5386	5387
56.	5471	5472	5473	5474	5475	5476	5477
57.	5561	5562	5563	5564	5565	5566	5567
58.	5651	5652	5653	5654	5655	5656	5657
59.	5741	5742	5743	5744	5745	5746	5747
60.	5831	5832	5833	5834	5835	5836	5837
61.	5921	5922	5923	5924	5925	5926	5927
62.	6011	6012	6013	6014	6015	6016	6017
63.	6101	6102	6103	6104	6105	6106	6107
64.	6191	6192	6193	6194	6195	6196	6197
65.	6281	6282	6283	6284	6285	6286	6287
66.	6371	6372	6373	6374	6375	6376	6377
67.	6461	6462	6463	6464	6465	6466	6467
68.	6551	6552	6553	6554	6555	6556	6557
69.	6641	6642	6643	6644	6645	6646	6647
70.	6731	6732	6733	6734	6735	6736	6737
71.	6821	6822	6823	6824	6825	6826	6827
72.	6911	6912	6913	6914	6915	6916	6917
73.	7001	7002	7003	7004	7005	7006	7007
74.	7091	7092	7093	7094	7095	7096	7097
75.	7181	7182	7183	7184	7185	7186	7187
76.	7271	7272	7273	7274	7275	7276	7277
77.	7361	7362	7363	7364	7365	7366	7367
78.	7451	7452	7453	7454	7455	7456	7457
79.	7541	7542	7543	7544	7545	7546	7547
80.	7631	7632	7633	7634	7635	7636	7637
81.	7721	7722	7723	7724	7725	7726	7727
82.	7811	7812	7813	7814	7815	7816	7817
83.	7901	7902	7903	7904	7905	7906	7907
84.	7991	7992	7993	7994	7995	7996	7997
85.	8081	8082	8083	8084	8085	8086	8087
86.	8171	8172	8173	8174	8175	8176	8177
87.	8261	8262	8263	8264	8265	8266	8267
88.	8351	8352	8353	8354	8355	8356	8357
89.	8441	8442	8443	8444	8445	8446	8447
90.	8531	8532	8533	8534	8535	8536	8537
91.	8621	8622	8623	8624	8625	8626	8627
92.	8711	8712	8713	8714	8715	8716	8717
93.	8801	8802	8803	8804	8805	8806	8807
94.	8891	8892	8893	8894	8895	8896	8897
95.	8981	8982	8983	8984	8985	8986	8987
96.	9071	9072	9073	9074	9075	9076	9077
97.	9161	9162	9163	9164	9165	9166	9167
98.	9251	9252	9253	9254	9255	9256	9257
99.	9341	9342	9343	9344	9345	9346	9347
100.	9431	9432	9433	9434	9435	9436	9437
101.	9521	9522	9523	9524	9525	9526	9527
102.	9611	9612	9613	9614	9615	9616	9617
103.	9701	9702	9703	9704	9705	9706	9707
104.	9791	9792	9793	9794	9795	9796	9797
105.	9881	9882	9883	9884	9885	9886	9887
106.	9971	9972	9973	9974	9975	9976	9977
107.	10061	10062	10063	10064	10065	10066	10067
108.	10151	10152	10153	10154	10155	10156	10157
109.	10241	10242	10243	10244	10245	10246	10247
110.	10331	10332	10333	10334	10335	10336	10337
111.	10421	10422	10423	10424	10425	10426	10427
112.	10511	10512	10513	10514	10515	10516	10517
113.	10601	10602	10603	10604	10605	10606	10607
114.	10691	10692	10693	10694	10695	10696	10697
115.	10781	10782	10783	10784	10785	10786	10787
116.	10871	10872	10873	10874	10875	10876	10877
117.	10961	10962	10963	10964	10965	10966	10967
118.	11051	11052	11053	11054	11055	11056	11057
119.	11141	11142	11143	11144	11145	11146	11147
120.	11231	11232	11233	11234	11235	11236	11237
121.	11321	11322	11323	11324	11325	11326	11327
122.	11411	11412	11413	11414	11415	11416	11417
123.	11501	11502	11503	11504	11505	11506	11507
124.	11591	11592	11593	11594	11595	11596	11597
125.	11681	11682	11683	11684	11685	11686	11687
126.	11771	11772	11773	11774	11775	11776	11777
127.	11861	11862	11863	11864	11865	11866	11867
128.	11951	11952	11953	11954	11955	11956	11957
129.	12041	12042	12043	12044	12045	12046	12047
130.	12131	12132	12133	12134	12135	12136	12137
131.	12221	12222	12223	12224	12225	12226	12227
132.	12311	12312	12313	12314	12315	12316	12317
133.	12401	12402	12403	12404	12405	12406	12407
134.	12491	12492	12493	12494	12495	12496	12497
135.	12581	12582	12583	12584	12585	12586	12587
136.	12671	12672	12673	12674	12675	12676	12677
137.	12761	12762	12763	12764	12765	12766	12767
138.	12851	12852	12853	12854	12855	12856	12857
139.	12941	12942	12943	12944	12945	12946	12947
140.	13031	13032	13033	13034	13035	13036	13037
141.	13121	13122	13123	13124	13125	13126	13127
142.	13211	13212	13213	13214	13215	13216	13217
143.	13301	13302	13303	13304	13305	13306	13307
144.	13391	13392	13393	13394	13395	13396	13397
145.	13481	13482	13483	13484	13485	13486	13487
146.	13571	13572	13573	13574	13575	13576	13577
147.	13661	13662	13663	13664	13665	13666	13667
148.	13751	13752	13753	13754	13755	13756	13757
149.	13841	13842	13843	13844	13845	13846	13847
150.	13931	13932	13933	13934	13935	13936	13937
151.	14021	14022	14023	14024	14025	14026	14027
152.	14111	14112	14113	14114	14115	14116	14117
153.	14201	14202	14203	14204	14205	14206	14207
154.	14291	14292	14293	14294	14295	14296	14297
155.	14381	14382	14383	14384	14385	14386	14387
156.	14471	14472	14473	14474	14475	14476	14477
157.	14561	14562	14563	14564	14565	14566	14567
158.	14651	14652	14653	14654	14655	14656	14657
159.	14741	14742	14743	14744	14745	14746	14747
160.	14831	14832	14833	14834	14835	14836	14837
161.	14921	14922	14923	14924	14925	14926	14927
162.	15011	15012	15013	15014	15015	15016	15017
163.	15101	15102	15103	15104	15105	15106	15107
164.	15191	15192	15193	15194	15195	15196	15197
165.	15281	15282	15283	15284	15285	15286	15287

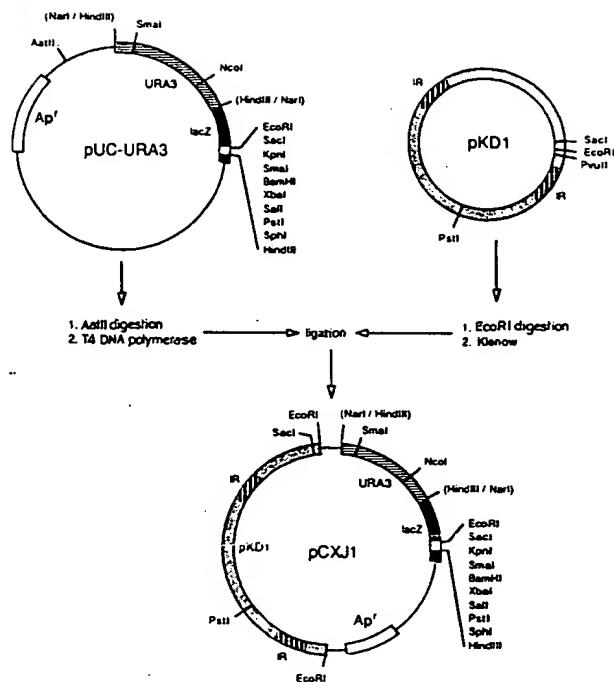


FIG. 9

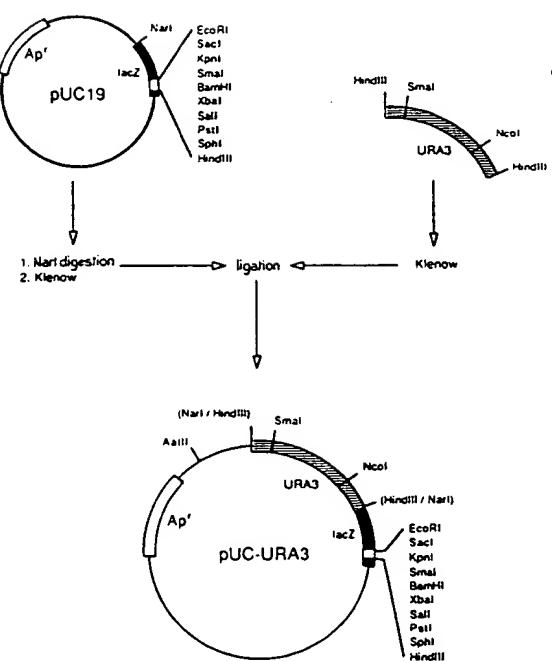
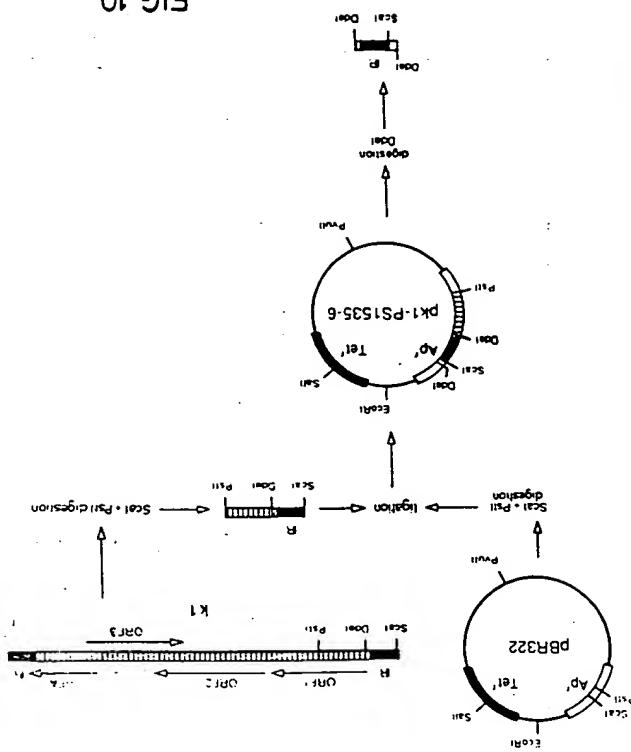


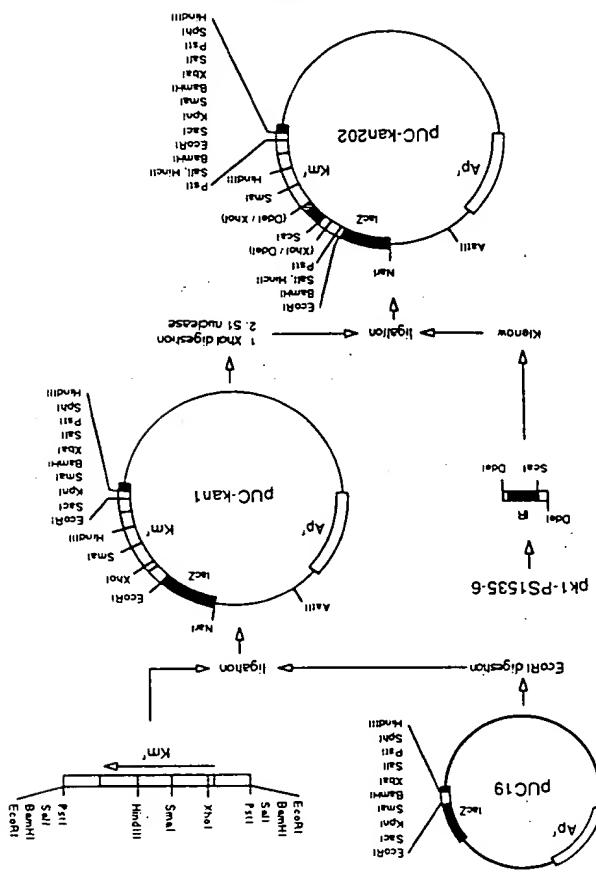
FIG. 8

FIG. 10



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FIG. 11



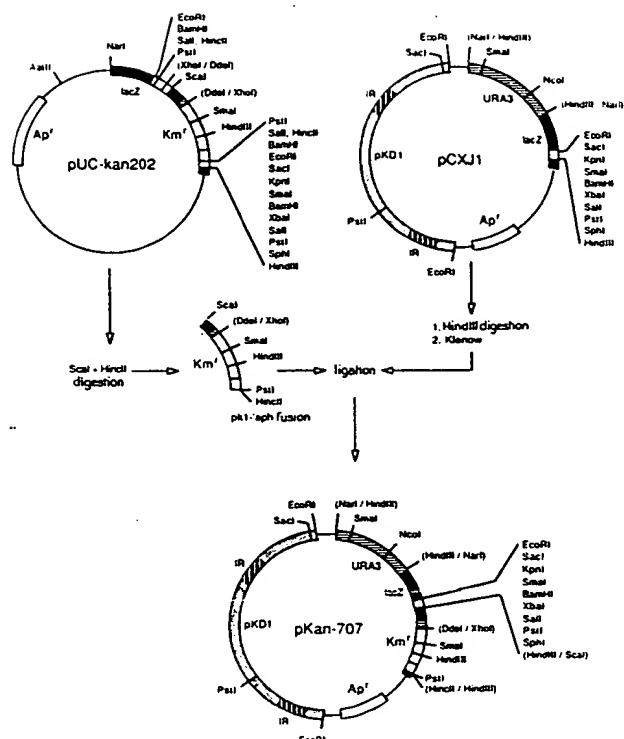


FIG.13

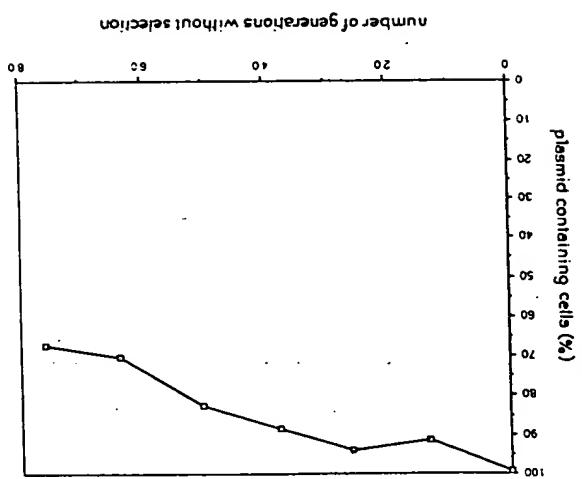
promoter pkl

5' - AGTACTAAAGTGAGATTATGGAAAGTTAGA:CTCTCCATTTTTTGCTTTTTTTTTTTTT
 ATATAGGTATTTTTTCAGTTATATCTTCCTGTATAACAGCTATTTTATATTAACTC
 TATTTTACACTTATGACCTATAAGTCATTTTATTACACATTTCCAACCTATAATAACAGAAATTAATT
 ATTAATTTAAAA | ATG GAT TTC AAA GAT AAG GCT TTA AAT GAT CTA AGG CGG CGA
 ORF1' ← → uph

TTA AAT TCC AAC ... - 3'

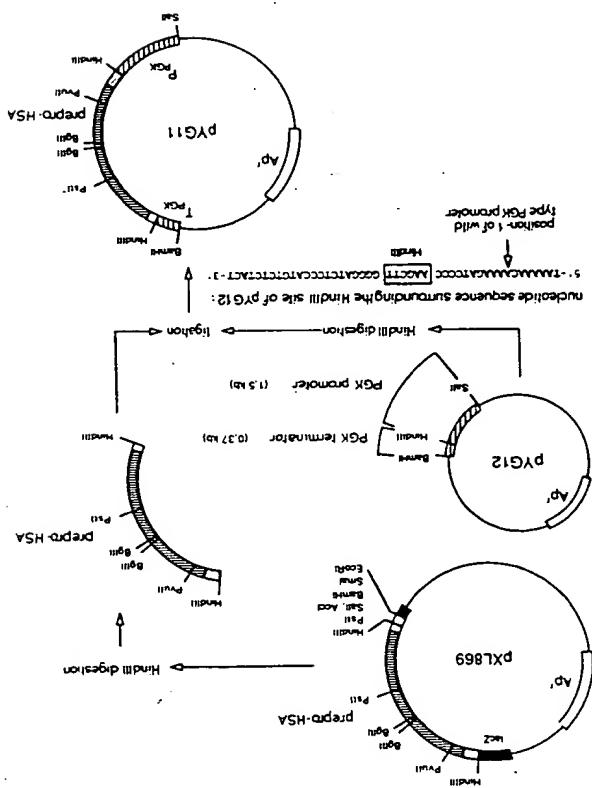
FIG. 12

FIG.14



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FIG.15



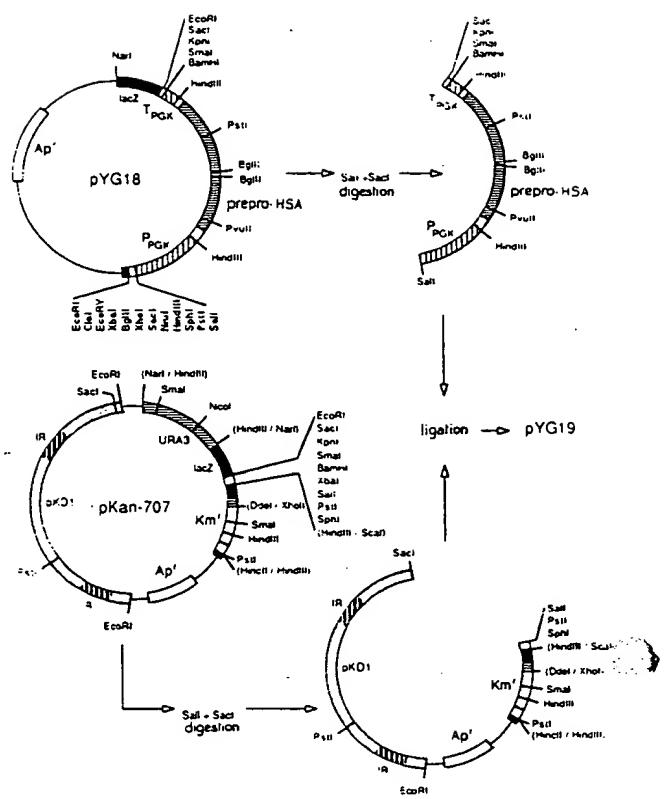


FIG. 17

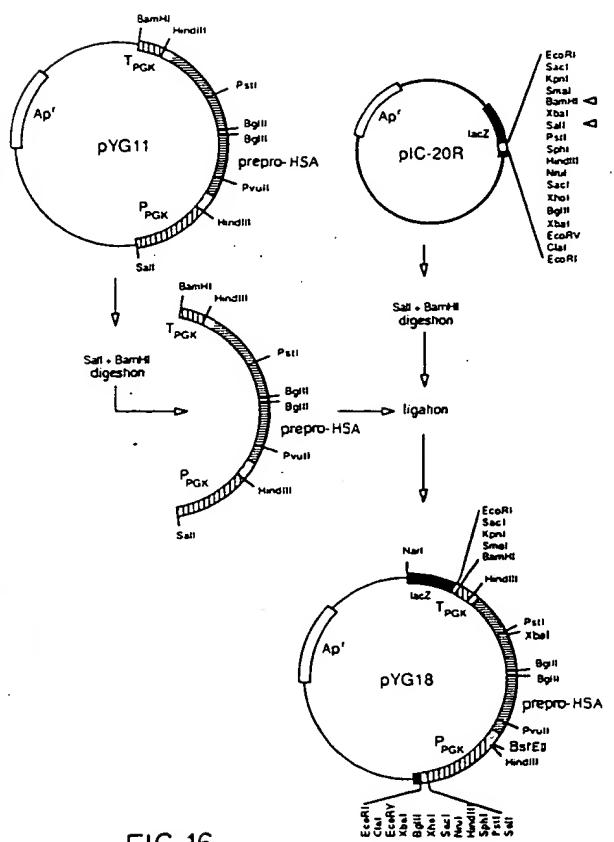
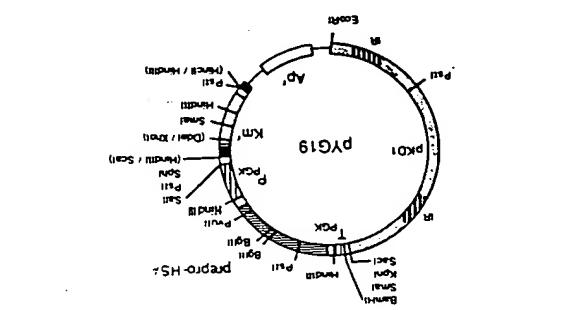
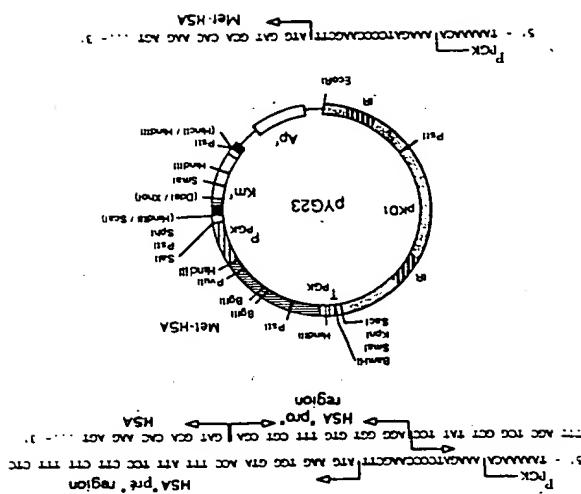


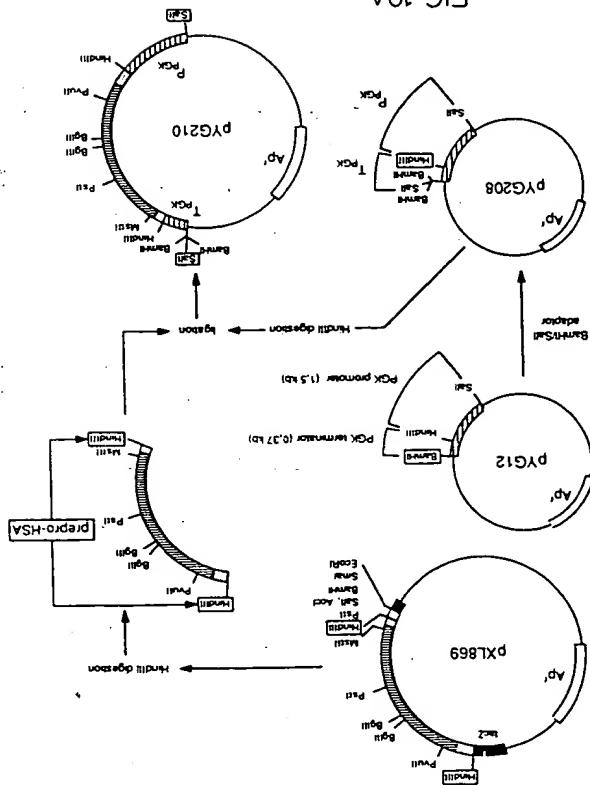
FIG. 16

FIG.18



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FIG.19A



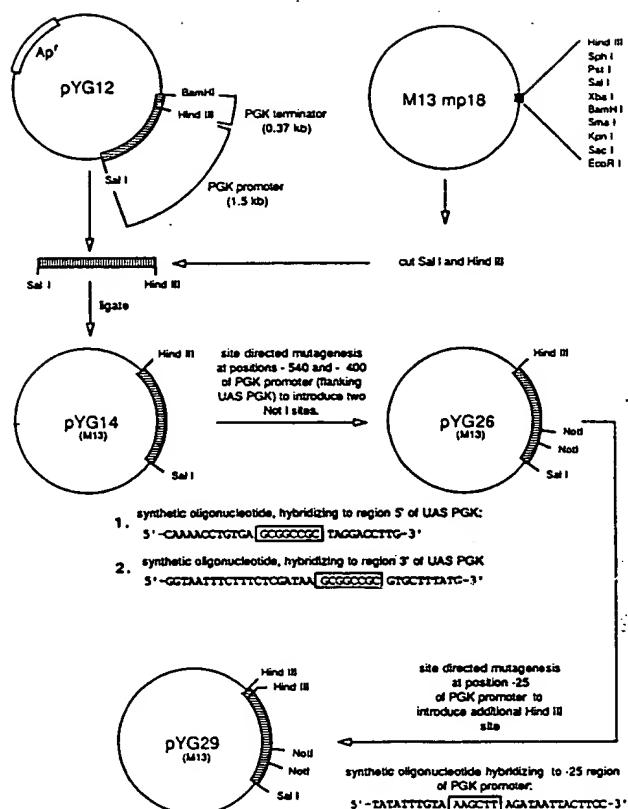


FIG. 20

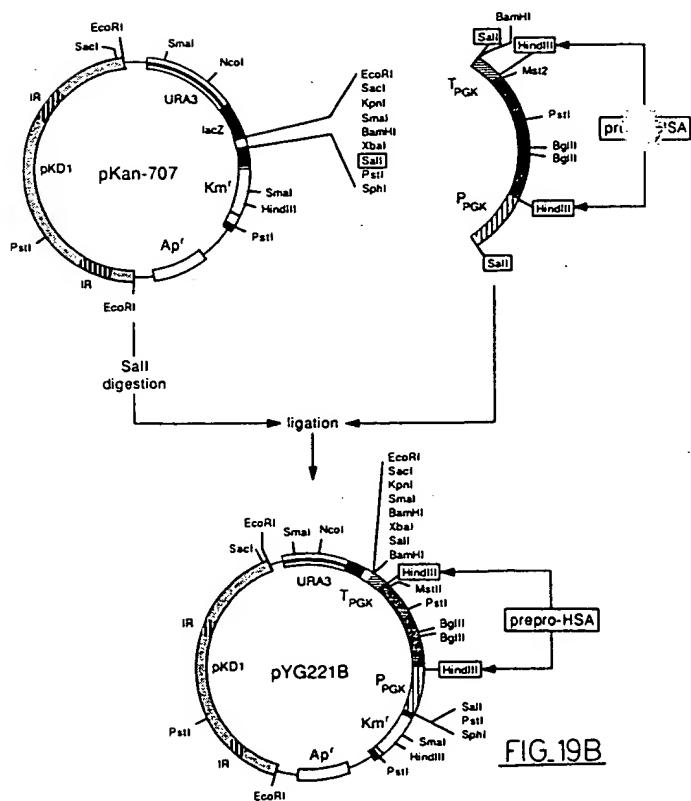
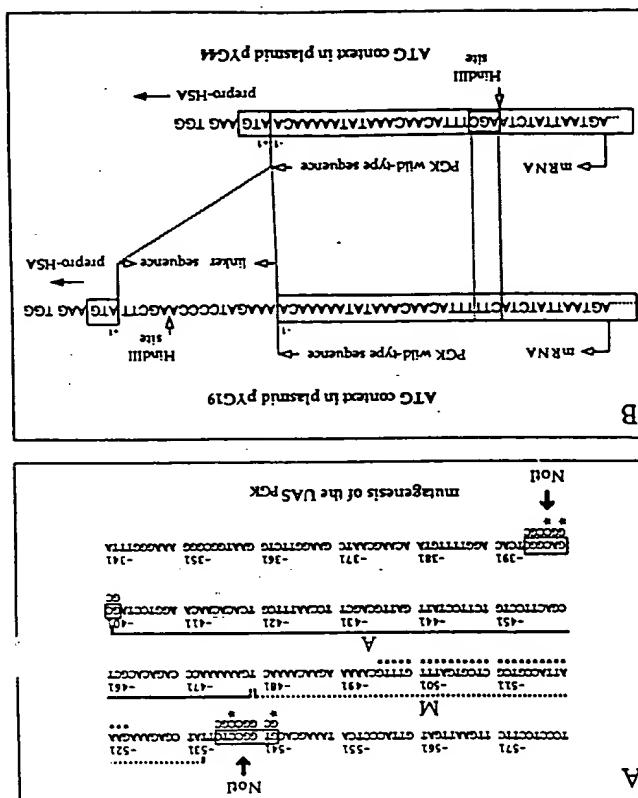


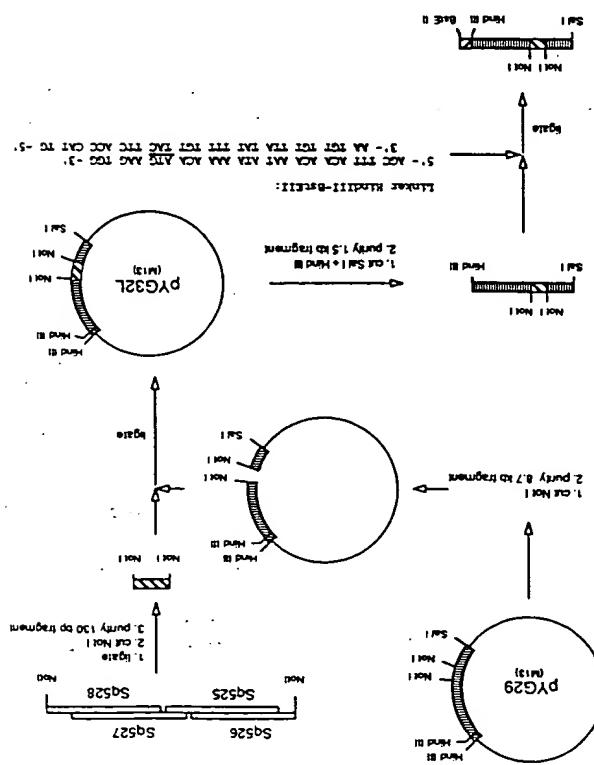
FIG. 19B

F16.21



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-1G-22



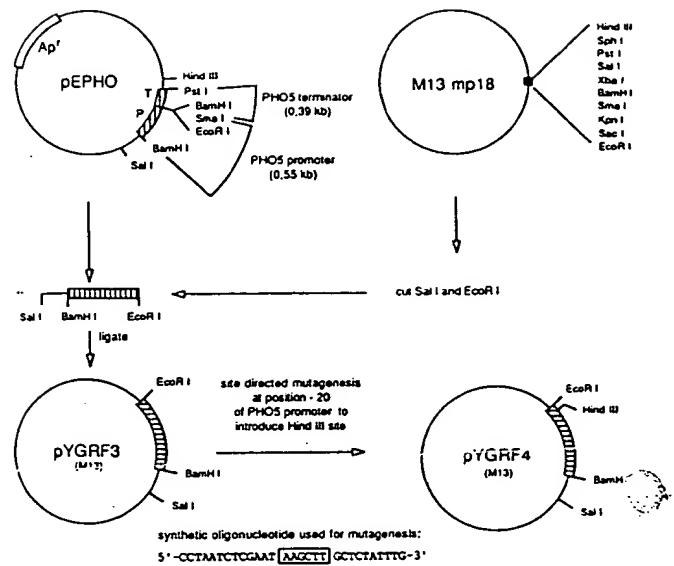


FIG. 24

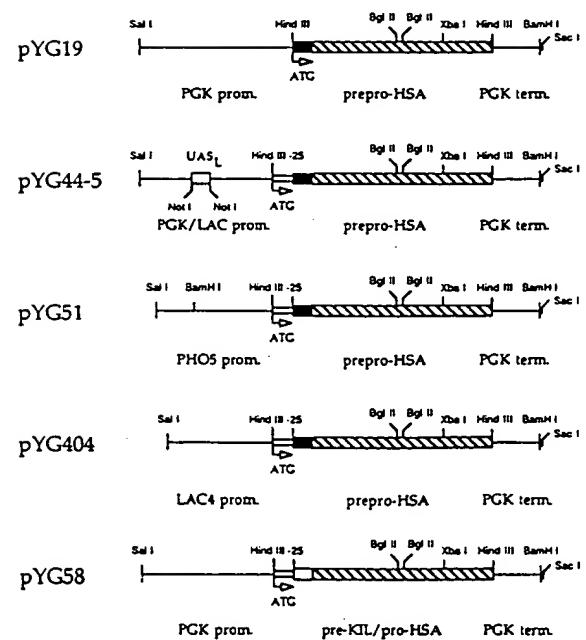
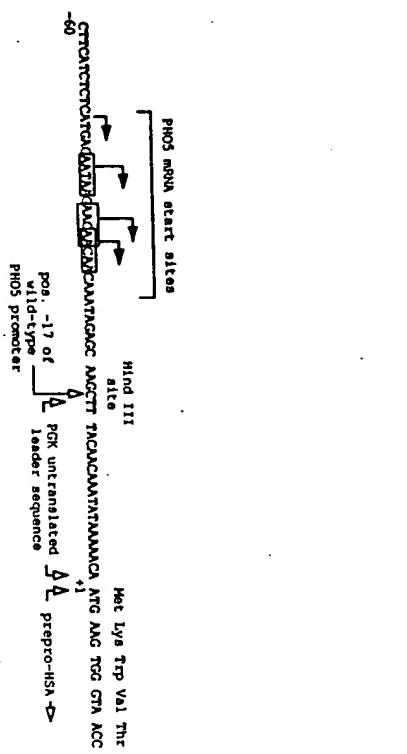


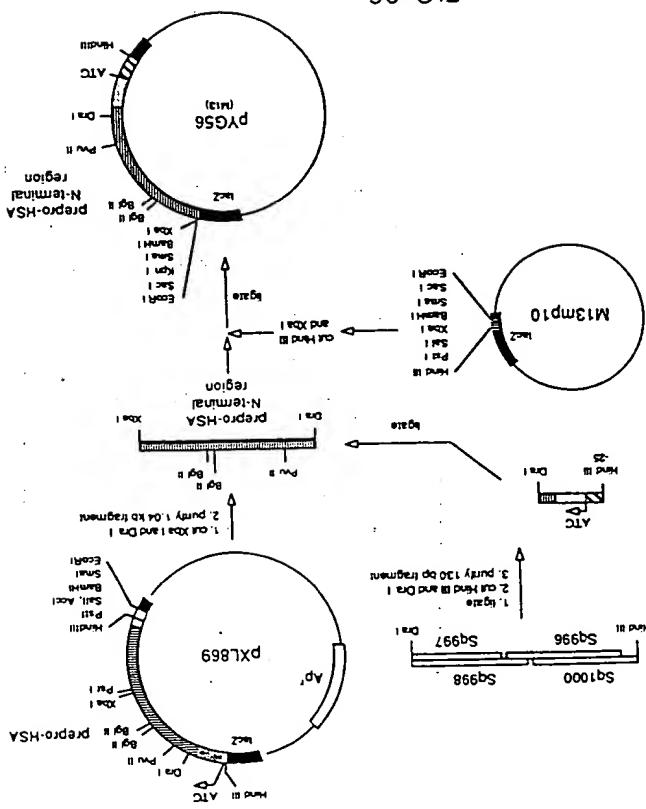
FIG. 23

FIG. 25



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F1G. 26



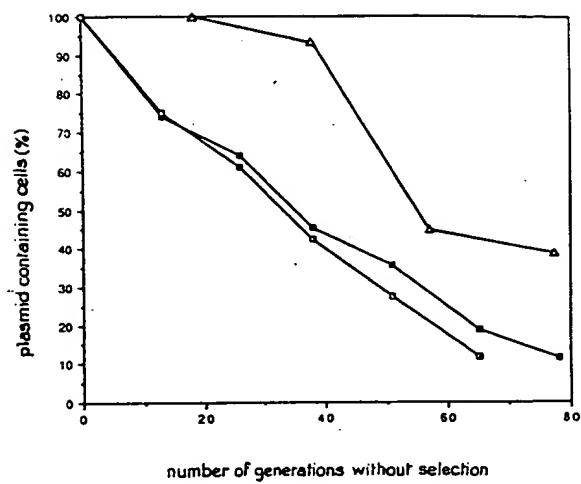


FIG. 28

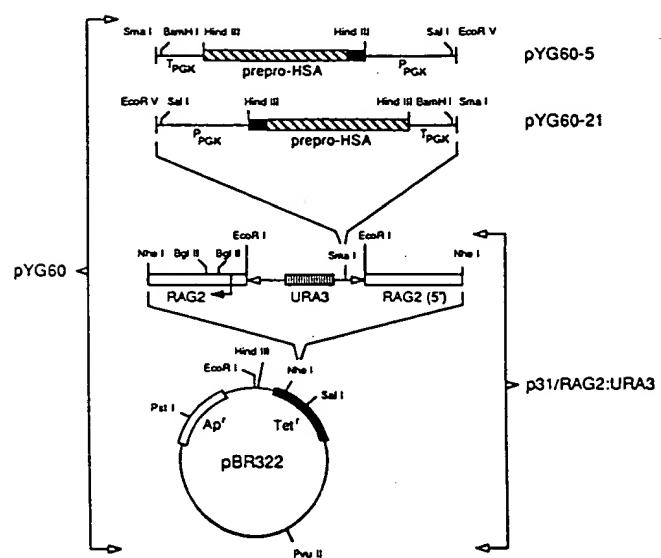


FIG. 27

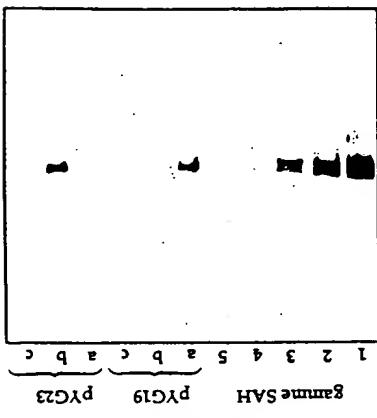


FIG. 30

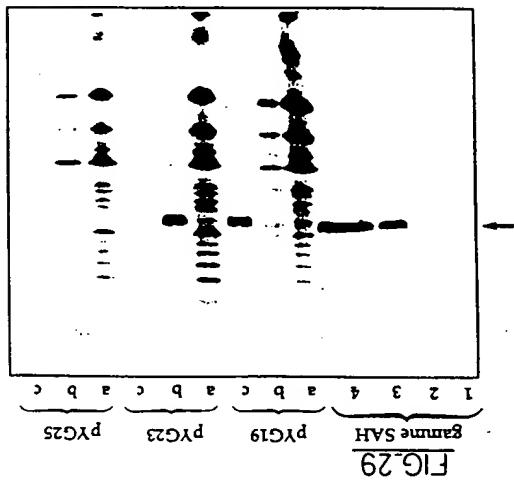
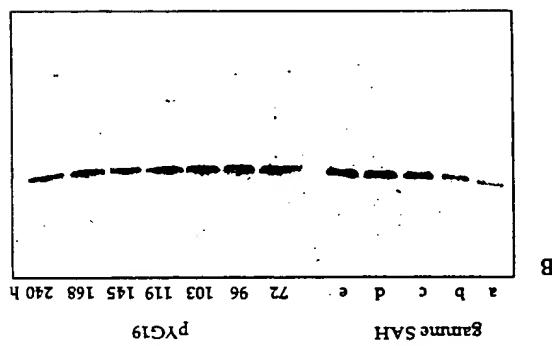


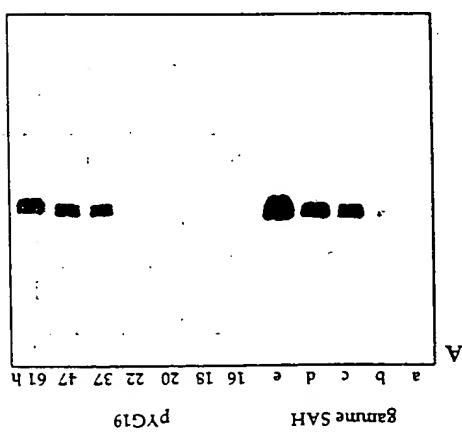
FIG. 29

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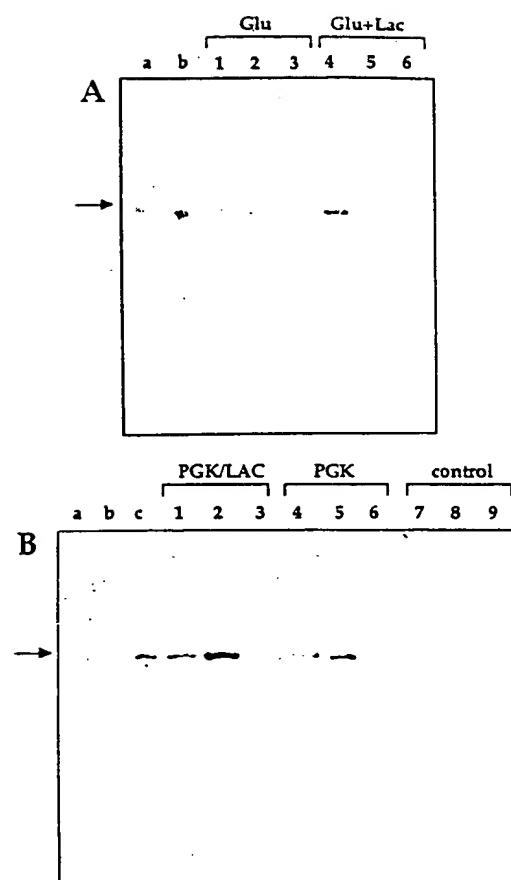
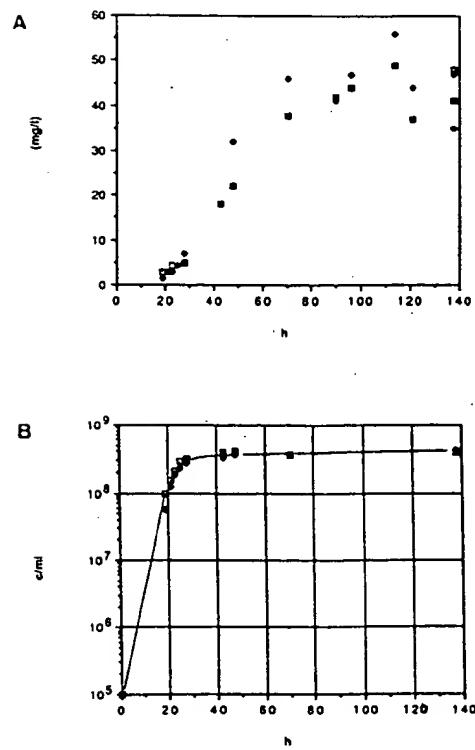
FIG. 31



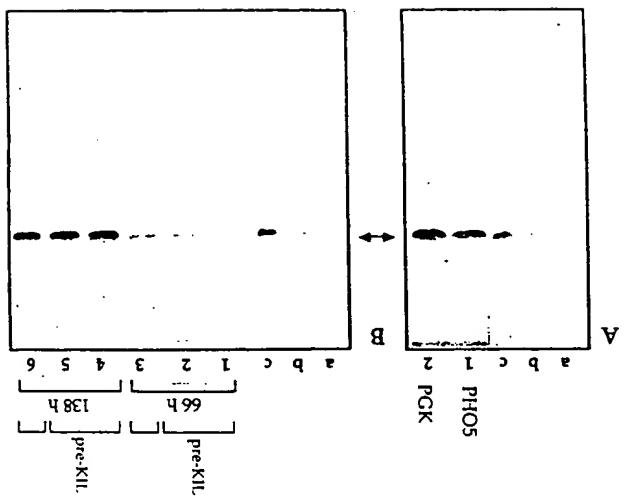
B
gamma-SAH pY/G19



A
gamma-SAH pY/G19

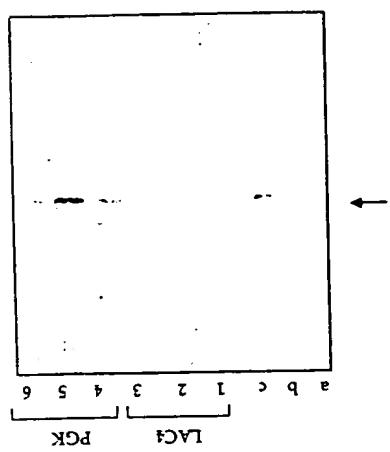
FIG. 33FIG. 32

FIG_34



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FIG_35



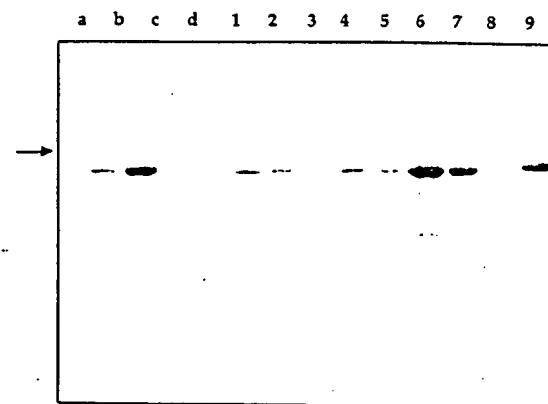
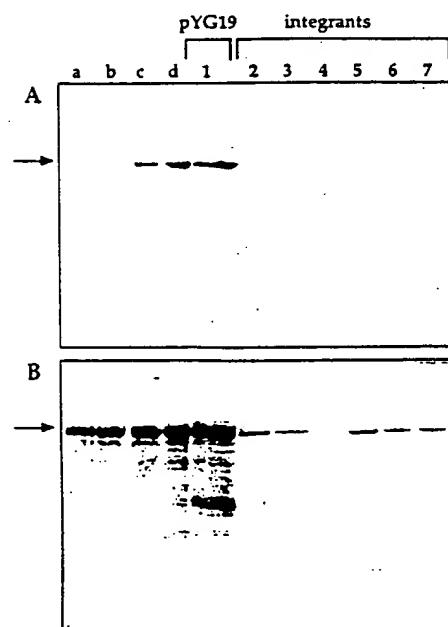
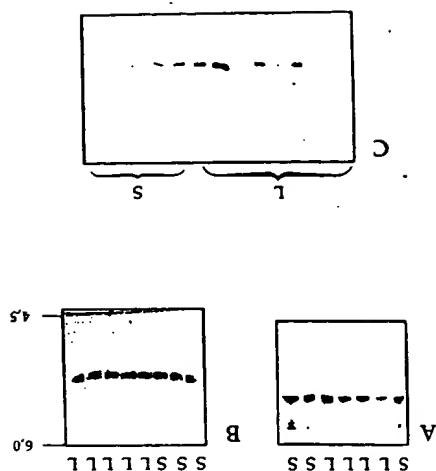
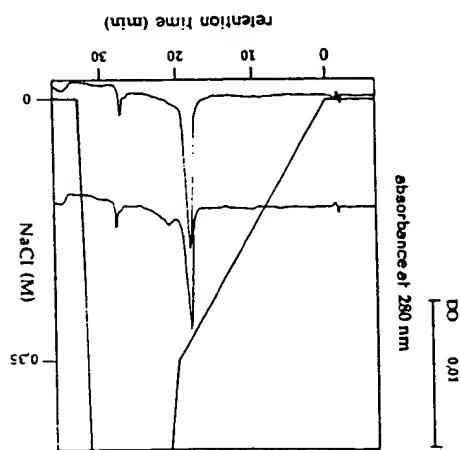
FIG_37FIG_36

FIG. 38



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FIG. 39



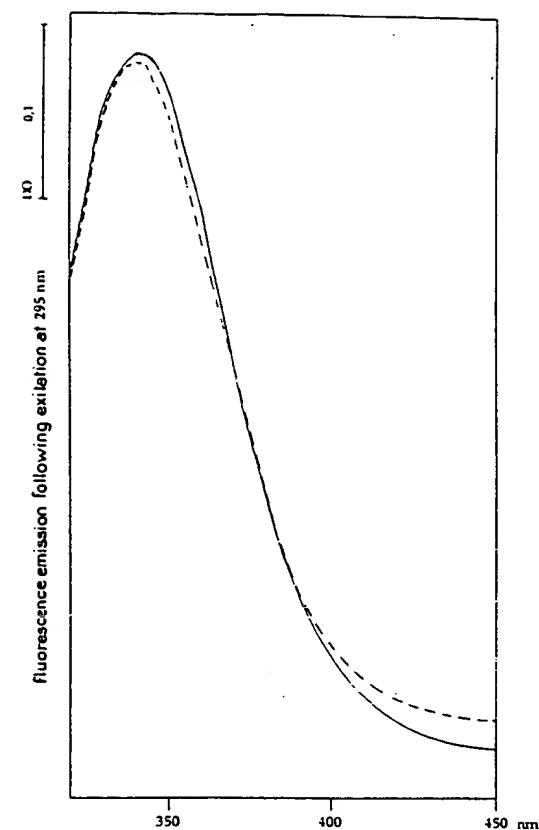
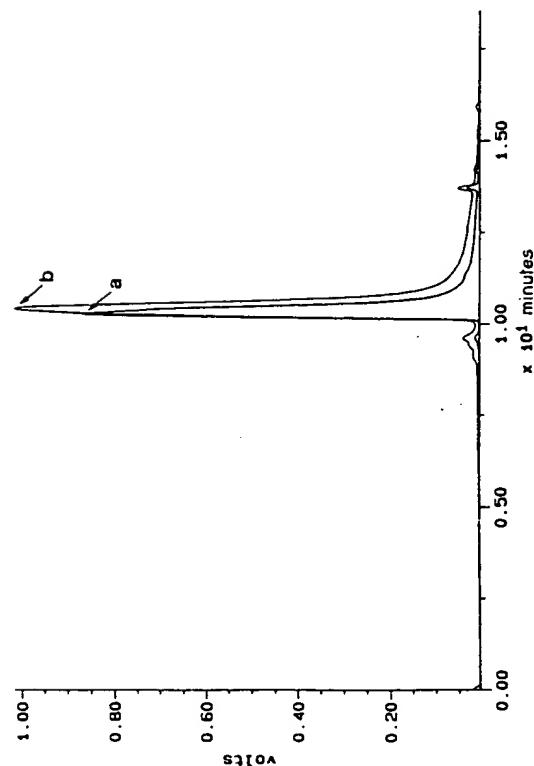
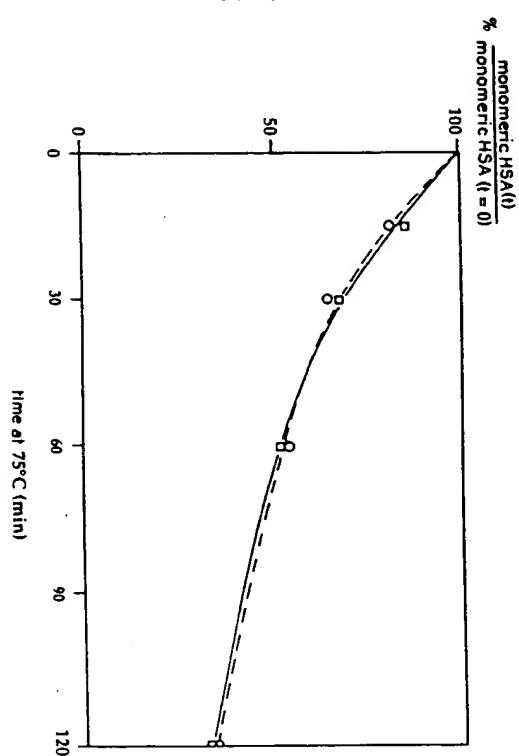
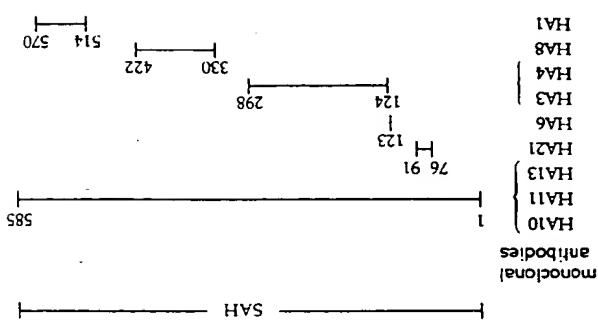
FIG.41FIG.40

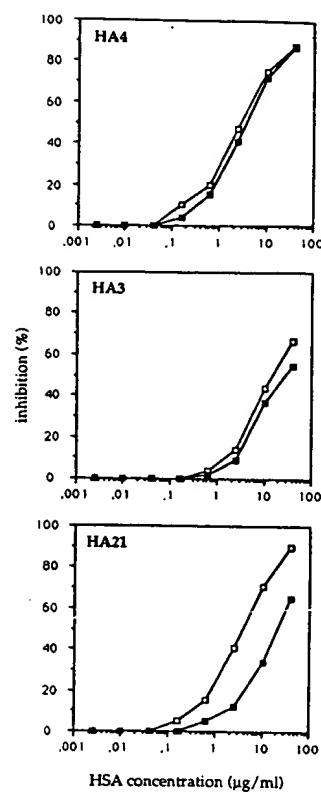
FIG. 42



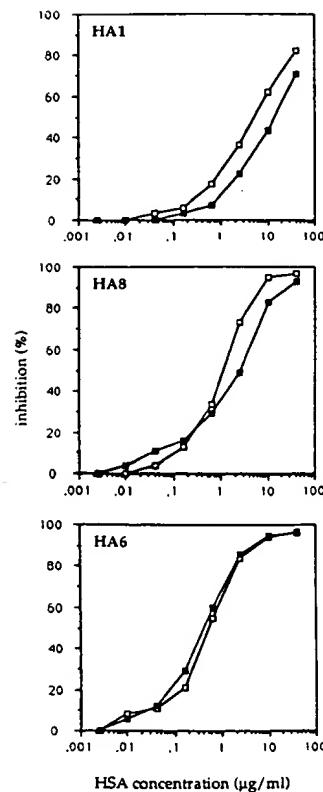
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FIG. 43





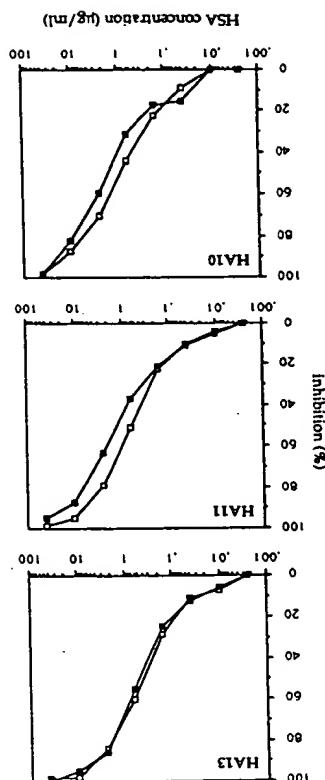
HSA concentration (µg/ml)

FIG_44B

HSA concentration (µg/ml)

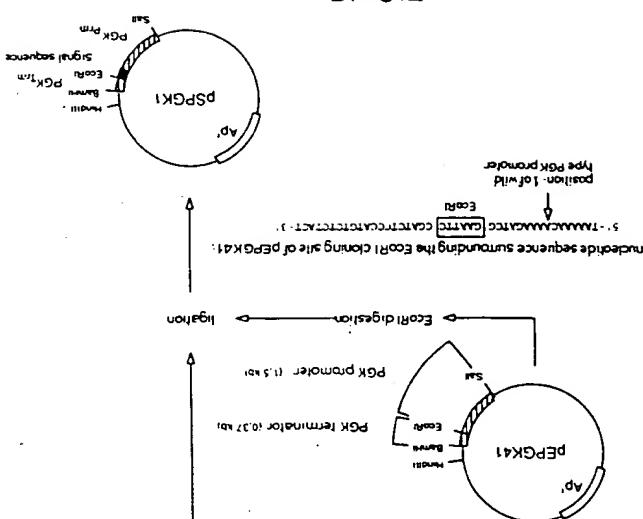
FIG_44A

FIG-LLC



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1645



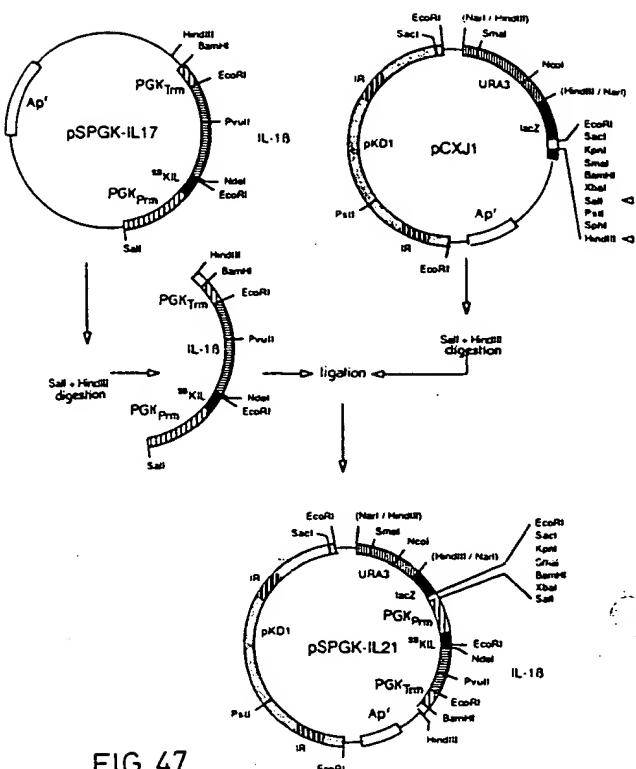


FIG. 47

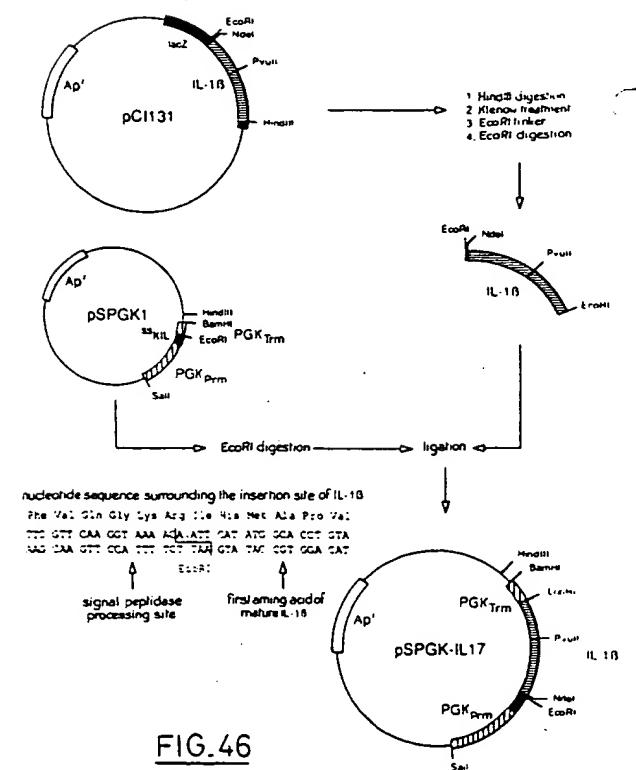
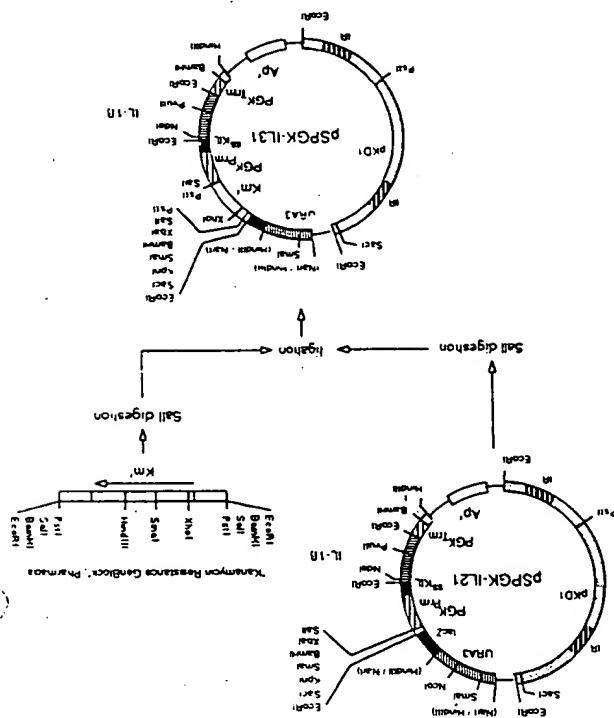


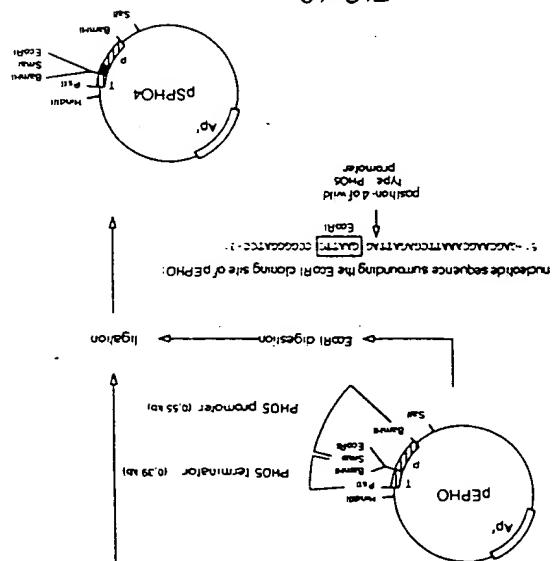
FIG. 46

FIG. 48



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F16.49



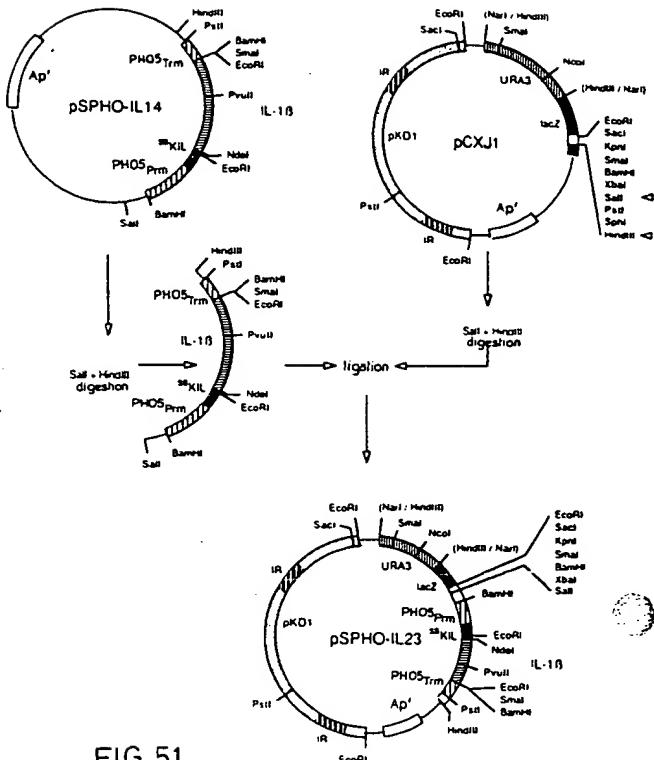


FIG. 51

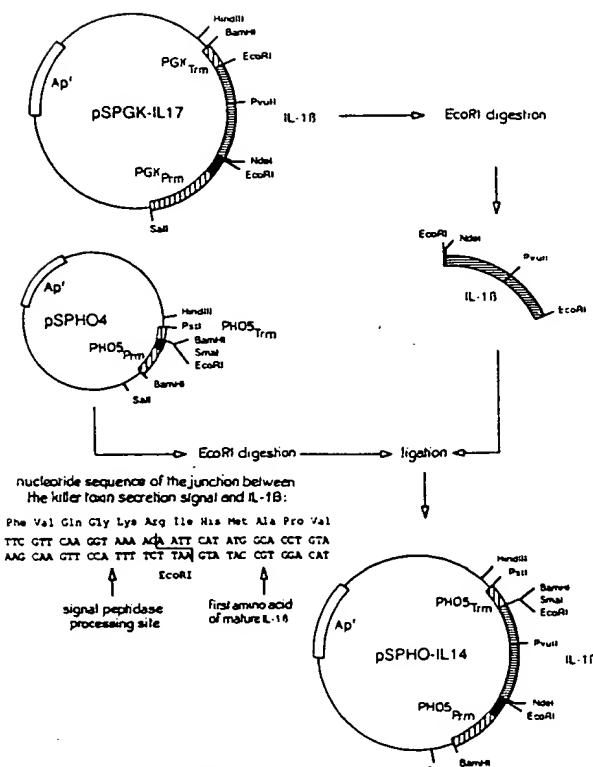
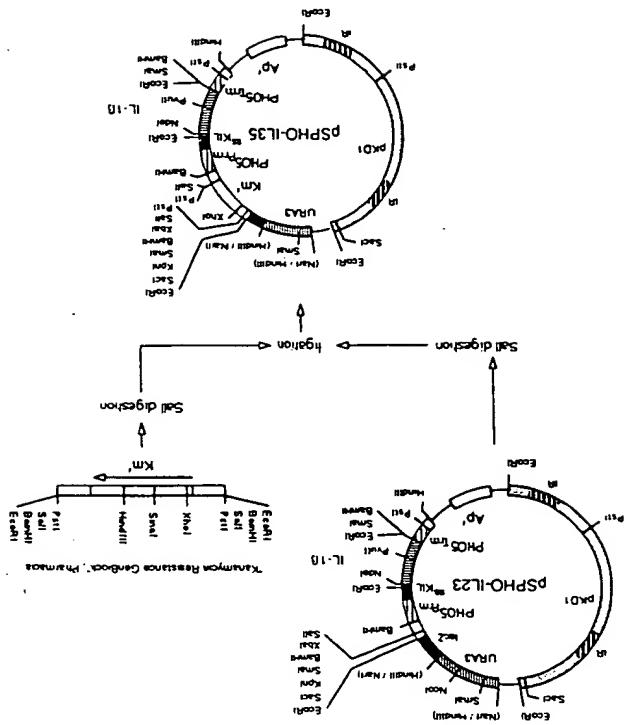


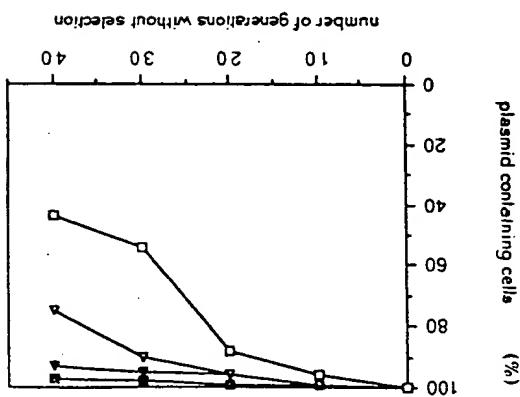
FIG. 50

FIG. 52



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FIG. 53



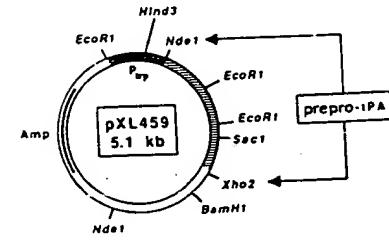
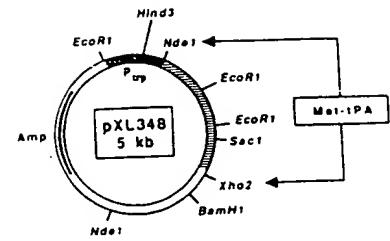


FIG.55

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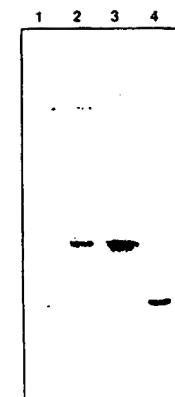


FIG.54

FIG.57

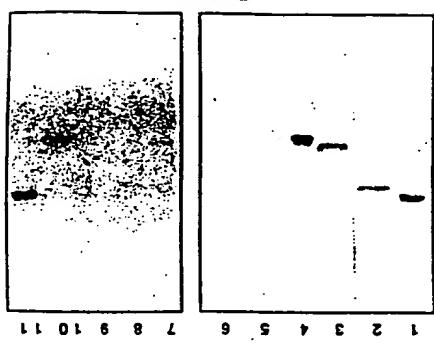
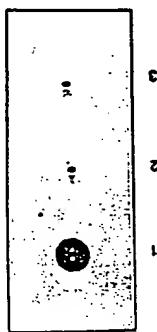


FIG.56



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